IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: Fronda, Christian L.
	)	
Woon-Lam Susan LEUNG, *et al.*	)	Art Unit: 1652
	)	
Application Serial No. 09/422,528	)	Confirmation No. 5652
	)	
Filed: October 21, 1999	)	Attorney's Docket No. GNE-0128A
	)	
For: **PROCESS FOR BACTERIAL PRODUCTION OF POLYPEPTIDES**	)	Customer No. 35489

FILED VIA EFS – AUGUST 17, 2009

DECLARATION OF MICHAEL W. LAIRD, Ph. D.

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I MICHAEL W. LAIRD, Ph.D. declare and say as follows: -

1. I obtained a B.S. in Biology from Iowa State University in 1991, a Ph.D. in Microbiology from Arizona State University in 1996, and completed postdoctoral training at Genentech, Inc. (CA) in 1999. Subsequently, I was employed by Human Genome Sciences with increasing levels of responsibilities and titles until 2005.

2. In 2005 I joined Genentech, Inc. as Senior Scientist where my current title is Associate Director/Late Stage Cell Culture.

3. I have extensive experience with cell culture processes for the recombinant production of heterologous polypeptides, including therapeutic antibodies and growth factors, in various host organisms, including bacterial hosts, in particular various *Escherichia coli* (*E. coli*) strains.

4. My Scientific Curriculum Vitae is enclosed as Exhibit A and forms part of this Declaration.

5. I have read and understand the disclosure and claims of the above-identified patent application.

6. I have also read and understand the Office Actions mailed on March 17, 2008 and February 17, 2009 in connection with the above-identified patent application and Applicants' response of December 17, 2008, and the following publications, cited in the Office Actions:

Hart et al., BIO/TECHNOLOGY Vol. 12, November 1994;

EP 0 155 189 (Wetzel et al.); and

Dien et al., Appl Environ Microbiol. 1997 May; 63(5):1689-95.

7. Claims 1-13 and 15-25 pending in this application concern a process for recovering refractile particles containing a heterologous polypeptide from bacterial periplasm in which the polypeptide is insoluble by coordinated expression of nucleic acid encoding the desired heterologous polypeptide and nucleic acid encoding a phage lysozyme, thereby releasing insoluble refractile particles from the peptidoglycan layer of the bacterial periplasm. Claim 1 states that "*expression of the nucleic acid encoding the phage lysozyme is induced by the addition of an inducer after about 50% or more of the heterologous polypeptide has accumulated.*" An example of the heterologous polypepides produced by the claimed process is human insulin-like growth factor-I (IGF-I), an exemplary lysozyme is T4 phage lysozyme and an exemplary inducer is arabinose.

8. I understand the patent Examiner's position to be that the invention claimed in this application is obvious over the combination of references listed in paragraph 6 of this Declaration, even though the references do not teach or suggest to wait until 50% or more of the human IGF-I has accumulated before inducing expression of the phage lysozyme. I further understand that the reason for this conclusion is that, according to the Examiner, one of ordinary skill in the art would have been motivated to wait until 50% or more of the human IGF-I or other representative heterologous protein has accumulated before inducing expression of T4 phage

lysozyme in order to obtain a greater yield of the heterologous protein, and would have had a reasonable expectation of success to achieve this goal.

9. The timing for the induction of the phage lysozyme is a very delicate balance between guaranteeing that the cells have achieved an appropriate concentration of the recombinant product prior to lysozyme production and ensuring that there is enough lysozyme at harvest so that its effectiveness is not severely diminished. It was known in the art at the time the present invention was made that high level production of recombinant proteins during fermentations is growth associated and occurs at its maximum rate during the exponential growth phase of the culture (Kim and Ryu, Biotechnol Bioeng, 38:1271-1279 (1991) (Exhibit B) and Gupta et al., J Biotechnol 68:125-134 (1999) (Exhibit C)). As bacterial cultures enter into stationary phase upon nutrient and energy depletion, the machinery responsible for recombinant protein production is depleted in favor of maintaining the cellular metabolic activity (i.e. eliminates non-essential activities to maintain viability) (Martin, Mol Microbiol 5:3-10, (1991) (Exhibit D); and Dedhia et al., Biotechnol Bioeng, 53:379-386 (1996) (Exhibit E)). The process disclosed and claimed in the present application requires the production of two recombinant proteins: human IGF-I and a phage lysozyme. Before the invention disclosed and claimed in this application it was not known whether after accumulation of 50% or more of human IGF-I, when the nutrient and the energy pools are depleted to the point where cell survival is primary, the remaining cellular machinery would be able to produce sufficient amounts of the second recombinant protein, the phage lysozyme, to be effective to perform its intended role. Therefore, while waiting until accumulation of at least 50% of human IGF-I might have been desirable in order to increase IGF-I production, it was unpredictable whether this measure would not defeat the purpose of the process as a whole, which requires the production of the phage lysozyme in an amount sufficient to release retractile particles containing IGF-I from the cellular matrix or cell wall.

10. Based on my experience with the expression of heterologous proteins in bacterial hosts, including *E. coli*, and in view of the explanation provided in paragraph 9 of this Declaration, it is my considered scientific opinion that at the time the present invention was made one of ordinary skill in the art would not have had a reasonable expectation that at the end

of a long fermentation process and after substantial IGF-I product accumulation the host could produce enough of the phage lysozyme to be effective.

11. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further, that these statements are made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent granted thereon.

Dated:_____ _____

MICHAEL W. LAIRD, Ph.D.

LIBC/3656624.1

EXHIBIT A

Michael W. Laird, Ph.D.

Late Stage Cell Culture
Process Research & Development
Genentech, Inc.
650-467-4596
mlaird@gene.com

Education

Post-Doctoral Research Fellow, Genentech, Inc.	1997-1999
Ph.D. in Microbiology, Arizona State University	1992-1996
B.S. in Biology, Iowa State University	1986-1991

Professional Experience

Genentech, Inc., South San Francisco, CA — 2005-Present
Sr. Scientist / Associate Director, Late Stage Cell Culture

Accomplishment highlights:

- Continue to lead the Nutropin v1.1 project as the CMC subteam leader (2006 – Present). To date, the team has:
  - Increased fermentation titers to ~1.5 g/L which links fermentation to recovery
  - Eliminated the use of specialized equipment (e.g. BEPEX freezer), open processing, safety issues, compliance concerns, long-range equipment purchases and 2-8°C processing.
  - Facilitated the ability to outsource the process
  - Developed an alternative bulk DS formulation that will permit bulk DS freezing
- Led the development of the 2H7 Phase 3 cell culture process (2005 – 2008)
  - Successfully completed two 12,000-L Phase 3 production campaigns (2006, 2008)
  - Authored or co-authored appropriate IND amendment sections
- Co-leader of a team that identified the antibody reduction mechanism and subsequent methods/procedures to prevent the reduction events at both the laboratory and manufacturing scales. This work resulted in the filing of a patent application.
- Responsible for the AP3 production at the CMO (Sicor) for the generation of DM-1
- Led the development of the Apo2L Phase 3 *E. coli* fermentation process
  - Increased fermentation titers from 6 g/L (Phase 2) to 8.3 g/L
  - Authored IND amendment sections relating to production host change for Phase 2
- Led the successful tech transfer & scale-up of the Phase 3 Apo2L process to the CMO (Boehringer-Ingelheim) in Vienna, Austria
  - Successfully completed a 4,000-L Phase 3 production campaign (2008)
  - Fermentation titers averaged > 9.5 g/L at scale
- Oversaw the VEGF Phase 3 *E. coli* fermentation process development (2005 – 2007)

-Increased fermentation titers from 2.5 g/L (Phase 2) to 9 g/L
-Successfully completed a 1,000-L Phase 3 production campaign (2007)

•Oversaw the development of the v114 Phase 2 & 3 cell culture processes (2005 – 2007)
-Increased the cell culture titers from 1 g/L (Phase 1) to 2.3 g/L (Phase 2)
-Successfully completed a 400-L Phase 2 production campaign (2007)
-Further process development increased cell culture titers to 3.5 g/L and achieved process lock prior to closing down the project.

•Completed the process characterization & validation studies for Lucentis (2005 – 2006)
-Co-developed the validation protocol to investigate the single CPP

•Authored *E. coli* section of QS00011 with input from Jane Gunson (2007 – 2008)

•Continue to lead numerous technology development initiatives including, but not limited to: *E. coli* host development optimization; novel glucose feeding algorithms; antibody reduction; antibody fucosylation; amino acid consumption in CHO cells; etc.

Miscellaneous contributions:

•Co-chair of the Process Development Research Program (PDRP) (2006 – 2007)
•Active member of Raw Materials Working Group (2006 – Present)
•Sponsor for the B3D Contamination Task Force (2006 – Present)
•Coordinator for the PR&D Postdoc Program (2007 – Present)
•Postdoc Mentor (2007 – Present)
•Active member of TEC (2007 – Present)
•Active member of the Glycosylation Task Force (GCTF; 2007 – Present)
•Active member of Bioprocess Design Forum (BPDF; 2008 – Present)
•Sponsor for B6 Controls Enhancement Task Force (2008 – Present)
•Active member of the Development Sciences Promotions & Appointments Committee (DSPAC) (2008 – Present)
•Co-leader of the Antibody Reduction Task Force with six subteams (2007 – Present)
•Active member of a team that is updating CHO & *E. coli* cell bank comparability protocols for future Regulatory submissions
•Active member of a team that is updating template CHO submission documents to incorporate the Quality by Design (QbD) strategy
•Active member of a team that developed a risk-based tool for implementing QbD within process development
•Continue to support GNE Regulatory Affairs for Lucentis by addressing questions from various regulatory agencies/countries & Novartis
•Since 2005, our group has authored over 25 technical documents which include technical references, technical reports, process descriptions and PC/PV protocols and summaries. There are approximately 15 more to be completed by the end of 2008.
•Our group co-authored 11 posters for the 2007 Asilomar meeting
•Our group continues to design projects to host coops and interns on a yearly basis

Human Genome Sciences, Inc., Rockville, MD — 2000-2005
Associate Director, Drug Manufacture & Development, CoGenesys Division — April 2005

Responsibilities:

•Fermentation process development (5-, 15- and 150-L).

-Led a group of nine that is responsible for developing scalable production and recovery processes for therapeutic candidates produced in *E. coli* and yeast.
-Generated product under GLP to be used in toxicology studies.
-Optimized fermentation conditions in conjunction with host/vector modifications and media for *E. coli* and yeast systems.
-Provided novel and general reagent protein production.

•Clinical production suite.
-Project lead to commission a cGMP production suite to make Phase 1 material. This work includes SOP and batch record generation as well as validation of equipment/facility.
-Led the fermentation/cell culture and recovery operations in the cGMP suite. Provided oversight of the purification activities.

•Quality and regulatory programs.
-Led a small team to design quality and regulatory systems/policies for the CoGenesys division, including training programs.

•Mammalian, yeast, and *E. coli* cGMP cell banking.
-Maintained all previous cGMP cell banking responsibilities for HGS and CoGenesys.
-Co-authored 'Cells at the Limit' protocol for the HGS lead candidate (Lympho-Stat B, mAb produced in NS0) and devised the testing strategy for the experiment.

•Continued molecular biology support for HGS and CoGenesys.
•Continued host strain and vector development for bacterial and yeast systems.
•Bacterial and yeast strain curator for entire company.
•Scientific liaison for numerous external collaborations to HGS and CoGenesys.

Section Head, Cell Banking & Reagent Group, Process Development 2003-2005

Responsibilities:

•Mammalian, yeast and *E. coli* cGMP cell banking.
-Prepared Chemistry, Manufacturing and Controls (CMC) and Investigational New Drug Application (IND) summaries for regulatory submissions relating to cell line development, production vector construction, and cell bank production and testing.
-Authored, reviewed, and approved batch records for cGMP master and working cell bank production.
-Requested the preparation of cGMP cell banks (master and working) at contract manufacturing organizations (CMOs) and in-house cell banking facilities. Generated 25 cGMP master and working cell banks since March 2003.
-Requested testing of cGMP cell banks (master and working) at contract testing organizations (CTOs).
-Evaluated and reported test results on cGMP cell banks.
-Developed cGMP testing methods for evaluating cGMP cell banks and end of production cells. Author, review, approve, and execute standard operating procedures (SOPs) related to testing cGMP cell banks. Utilize similar testing for accession cell banks.
-Documented cGMP cell bank preparation and testing via peer reviewed technical reports.

-Performed equivalency studies on yeast and *E. coli* master/working cGMP cell banks and report the data in peer reviewed technical reports. Orchestrate mammalian master/working cGMP cell bank equivalency studies amongst groups within Process Development.
-Documented cell line development, production vector construction, and accession cell bank preparation and testing via peer reviewed technical reports.
-Reviewed all technical reports related to mammalian, yeast, and *E. coli* cell banking.
-Authored company policies covering cell bank production, testing, characterization, storage, and stability.
-Performed formal facility audits of cell bank CMO/CTOs with HGS' QA group for cell bank production and testing.
-Investigated deviations related to cGMP cell bank production and testing. Develop and implement short- and long-term corrective action plans.
-Investigated aberrant cGMP test results. Develop new client specific protocols with CTOs to eliminate and prevent product or cell line related assay interferences.
-Developed an understanding of the regulatory guidelines.

•Generated *E. coli* and yeast accession cell banks for cGMP master cell banking.
•Host strain and vector development for *E. coli.*
•Host strain and vector development for *S. cerevisiae.*
•Performed small-scale purification of novel proteins.
•Provided reagent support for Research.
•Bacterial and yeast strain curator for the company.
•Directly managed a group of five that was responsible for purifying reagent grade proteins to support internal use in various research, GLP, and cGMP assays. Included general characterization via RP- and SEC-HPLC, N-terminal protein sequencing, and endotoxin analyses.
•Total, directly managed a group of nine (plus, up to four interns) performing genetic characterization assays, molecular genetics, molecular biology, host strain and plasmid vector development in *E. coli* and yeast, fermentation (5-L), cell culture, small-scale recovery, purification, and other related cell banking responsibilities. In addition, this group authored and reviewed SOPs and batch records relevant to cell bank production and testing, and reagent protein purification.

Accomplishment highlights:

•Developed a novel positive control for the detection of minute virus of mice (MVM) that eliminates false positive results.
•Coordinated the cGMP-qualification of the MVM-PCR method using infectious MVM at a CTO.
•Designed and started-up a cell culture laboratory. Actively culturing NS0, CHO, Vero, and 3T3 cell lines in T-flasks and shake flasks.
•Propagated numerous *Mycoplasma* species to serve as controls for detection methods and exclusion/elimination experiments (ongoing). Purposely infect cell culture lines and monitor contamination.
•Developed a novel positive control for use in a sensitive PCR-method for the detection of *Mycoplasma* in cell culture.

•Continued to optimize a novel, robust alternative promoter system for use in *S. cerevisiae*.

Sr. Scientist I, Cell Banking & Industrial Microbiology, Process Development 2002-2003

Responsibilities:

•Developed a cell bank program.
•Host strain and vector development for *E. coli*.
•Host strain and vector development for *S. cerevisiae*.
•Small-scale (5-L) fermentation process development for therapeutic candidates.
•Small-scale purification of novel proteins.
•Provide reagent support for Research.
•Bacterial and yeast strain curator for the company.
•Managed a group of 13 (plus, up to four interns) performing genetic characterization assays, molecular genetics, molecular biology, host strain and plasmid vector development in *E. coli* and yeast, fermentation (5-L), cell culture, small-scale recovery, purification, and other related cell banking responsibilities. In addition, this group authored and reviewed SOPs and batch records relevant to cell bank production and testing, and reagent protein purification.

Accomplishment highlights:

•Coordinated a small group of my direct reports to develop a cell bank program.
•Part of a cross disciplinary team (QA, QC, Regulatory Affairs, Manufacturing, Process Development) that defined required cell bank testing requirements (master and working cell bank release standards) for the development areas and manufacturing facilities. This same team defined cell bank specifications.
•Invited and hosted Cell Bank Consultant, Dr. Marshall Dinowitz (Pacific BioDevelopment) to critically review our cell bank program.
•Designed and set-up a cGMP laboratory for genetic stability testing of mammalian, yeast, and *E. coli* systems.
•Developed a sensitive and robust cGMP method to detect MVM from cell banks and large-scale production bioreactors.
•Developed cGMP cell bank and end of production cell assays for mammalian (copy number and restriction mapping by Southern blotting), yeast (copy number and restriction mapping by Southern blotting; plasmid retention; selectable marker; expression cassette rescue), and *E. coli* (copy number and restriction mapping by Southern blotting; plasmid retention; selectable marker; bacteriophage testing) systems. Developed the methods, authored SOPs, and qualified some of the methods (qualifications are ongoing).
•Developed cGMP methods to isolate production plasmids from mammalian, yeast, and *E. coli* cell banks and end of production cells to support cGMP DNA sequencing.
•Developed a novel fungal promoter system for heterologous protein production in *S. cerevisiae*. Propagated numerous fungi and isolated genomic DNA to use as template DNA for promoter construction.
•Developed an inducible promoter system (methionine depletion) for the production of human serum albumin (HSA)-fusion proteins in *S. cerevisiae*.
•PA production. Utilized a codon-optimized *Bacillus anthracis* Protective antigen (PA) gene and optimized protein production and purification conditions to produce the PA

protein in *E. coli* from 5- and 50-L defined media fermentations. Generated >370 mg/L of soluble, biologically active, pure PA. Literature exhibited yields of only 500 µg/L of soluble PA. Product was used as an antigen that led to the development of Abthrax, a monoclonal antibody for the prevention and treatment of Anthrax infections.

•EF production. Utilized a codon-optimized *Bacillus anthracis* Edema factor (EF) gene and optimized protein production and purification conditions to produce the EF protein in *E. coli* from 5-L fermentations. Generated >2.3 g/L of soluble, biologically active, pure EF. Literature exhibited yields of 2.5 mg/L of soluble EF. Used as a reagent in determining the neutralizing activity of antibodies against Anthrax Protective antigen by Edema factor-mediated cAMP-induction bioassay.

•LF production. Utilized a codon-optimized *Bacillus anthracis* Lethal factor (LF) gene and optimized protein production and purification conditions to produce the LF protein in *E. coli* from 5-L fermentations. Generated >1.3 g/L of soluble, biologically active, pure LF. Literature exhibited yields of only 20 mg/L of soluble LF. Used as a reagent for the development of neutralizing antibodies against Anthrax Protective antigen.

•Produced and purified hundreds of mg of F1 and V Plague antigens from *E. coli* that were used as antigens in the development of a monoclonal antibody for the treatment of Plague infections. These purified components were also used in *in vitro* and *in vivo* models.

•Continued to generate *E. coli* hosts for the production of heterologous proteins. In addition, generated yeast production hosts with similar useful characteristics that included protease deficiencies (plus, various combinations), co-expression of chaperones, physiological alterations, etc.

Scientist, Industrial Microbiology, Process Development 2000-2002

Responsibilities:

•Host strain and vector development for *E. coli*.

•Host strain and vector development for *S. cerevisiae*.

•Small-scale (5-L) fermentation process development for therapeutic candidates.

•Small-scale purification of novel proteins.

•Provide reagent support for Research.

•Became the bacterial and yeast strain curator for the company.

•Managed a group of six performing molecular genetics, molecular biology, host strain and plasmid vector development in *E. coli* and yeast, fermentation (5-L), small-scale recovery, and purification.

Accomplishment highlights:

•Successfully expressed the homo-trimeric, human BLyS protein in the periplasm of *E. coli*. BLyS stimulates immune system cells called B cells to mature into plasma B cells, which produce antibodies. Initial yield were ~30 mg/L of soluble BLyS from defined fermentations. Through expression vector and host optimization, as well as the development of a scaleable fermentation process, a yield >500 mg/L of biologically active, soluble BLyS was obtained. In addition, the initial 30 mg/L yield required an extremely costly affinity capture step. At >500 mg/L, we developed a conventional purification process that utilized standard, scaleable, cost-effective resins and eliminated the need for the affinity resin capture step.

•Developed and optimized a method without DNA amplification to rescue the expression cassette from the production plasmid in *S. cerevisiae*. Once the production plasmid is transformed into yeast it loses the *E. coli* origin of replication and its antibiotic resistance marker.
•Cloned, expressed, and purified mg quantities of >20 soluble, novel human proteins from *E. coli* shake flask and fermentation cultures.
•Generated *E. coli* hosts for the production of heterologous proteins. Features of these hosts included protease deficiencies (plus, various combinations), bacteriophage resistance, co-expression of chaperones, physiological alterations, etc.
•Designed searchable electronic database (Filemaker Pro) to catalogue baterial and yeast host strains, as well as plasmid vectors.
•Bacterial and yeast strain curator for the company.

Proteinix/IGEN International, Inc., Gaithersburg, MD 1999-2000
Scientist
•Primary goal was to develop a technology platform for the production of heterologous peptides and proteins in *Escherichia coli*.
•Host strain and vector development for protein fusion technology.
•Provided molecular biology, bacterial genetics, and physiology technical support to Proteinix, Inc., and IGEN International, Inc.
•Supported IGEN assay technologies by producing and purifying numerous reagent proteins, including large quantities of HIV Integrase.
•Managed and evaluated the research projects of one direct report.

Genentech, Inc., South San Francisco, CA 1997-1999
Post-Doctoral Research Fellow, Process Sciences Department
Mentor: Dr. John C. Joly
•Project goal was to discover novel factors that influence heterologous protein production in *Escherichia coli* using genetic selection schemes.
•Provided general molecular biology, bacterial genetics, and physiology support for the Process Sciences Department.

Arizona State University, Tempe, AZ 1992-1996
Research Associate, Department of Microbiology
Mentor: Dr. Rajeev Misra
•Research was conducted on outer membrane protein biogenesis in *E. coli*. The ultimate goal of this work was to elucidate the assembly and targeting pathway of outer membrane proteins using a variety of genetic and biochemical approaches.

Genencor International, Inc., Cedar Rapids, IA 1991-1992
Bio-Process Technician
•Part of a team that guided the enzyme production & recovery unit of a large-scale production facility through start-up and manufacturing phases (95,000-L fermentors and associated recovery train).
•Developed, authored, reviewed, and executed SOPs and was cGMP trained.

Skills

•Knowledge of regulatory guidelines, particular those relating to cell bank production, testing, characterization, and stability.

•Proficient in a wide range of scientific techniques and methods including, but not limited to: microbial genetic transfers and manipulations, gene mapping and cloning, mutation identification, DNA mutagenesis, vector construction and development, genomic library construction, cDNA cloning and expression, cDNA library construction, protein expression/optimization and analysis, PAGE, Westerns, RNA and mRNA isolation, Northerns, Southerns, ELISAs, cell based assays, pulse-chase technology, gradient centrifugation, physiological assays, PCR, QPCR, DNA sequencing, bioinformatics, lipid extraction and examination, thin layer chromatography, purification principles, assay development, microbial fermentation, cell culture, mycoplasma propagation and detection, mammalian, yeast, and bacterial cell banking (GLP & cGMP), genetic stability testing related to mammalian, yeast, and bacterial cell banking (GLP & cGMP), process development, and large-scale bio-process manufacturing methods.

•Experienced with both Windows and Macintosh software, including Microsoft Word, WordPerfect, Claris Draw, Filemaker Pro, Powerpoint, Excel, JMP, MATLAB, Sigma Plot, Design Cad, DNAMAN, Sequencher, and Microsoft Internet Explorer and Netscape Communicator.

Editorial Board

Protein Expression and Purification (2007 – present)

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•*Journal of Bacteriology*
•*Microbial Cell Factories*
•*Protein Expression and Purification*
•*Vaccine*

Publications

1. Blain K.Y., W. Kwaitkowski, Q. Zhao, D. LaFleur, C. Naik, T.-W. Chun, T. Tsareva, P. Kanakaraj, **M.W. Laird**, R. Shah, L. George, I. Sanyal, P.A. Moore, B. Demeler & S.

Choe (2007) Structural and Functional Characterization of CC Chemokine CCL14. *Biochemistry* 46:10008-10015.

2. Sherer K., Y. Li, X. Cui, X. Li, M. Subramanian, **M.W. Laird**, M. Moayeri, S.H. Leppla, Y. Fitz, J. Su & P.Q. Eichacker (2007) Fluid support worsens outcome and negates the benefit of protective antigen-directed monoclonal antibody in a lethal toxin-infused rat *Bacillus anthracis* shock model. *Crit Care Med* 35:1560-1567.

3. Cui X., Y. Li, X. Li, **M.W. Laird**, M. Subramanian, M. Moayeri, S.H. Leppla, Y. Fitz, J. Su, K. Sherer & P.Q. Eichacker (2007) *Bacillus anthracis* edema and lethal toxin have different hemodynamic effects but function together to worsen shock and outcome in a rat model. *J Infect Dis* 195:572-580.

4. Joly, J.C. & **M.W. Laird** (2007) Practical Applications for Periplasmic Protein Accumulation, IN *The Periplasm*. Edited by M. Ehrmann, ASM Press, Washington, D.C.

5. Gwinn, W., M. Zhang, S. Mon, D. Sampey, D. Zukauskas, C. Kassebaum, J.F. Zmuda, A. Tsai & **M.W. Laird** (2006) Scalable Purification of *Bacillus anthracis* Protective Antigen from *Escherichia coli*. *Protein Expr and Purif* 45:30-36.

6. Zmuda, J.F., L. Zhang, T. Richards, Q. Pham, D. Zukauskas, J.L. Pierre, **M.W. Laird**, J. Askins & G.H. Choi (2005) Determination of Neutralizing Activity of Antibodies Against Anthrax Protective Antigen by Edema Factor Mediated cAMP-Induction Bioassay. *J Immunol Methods* 298:49-62.

7. Solow, S.S., J. Sengbusch & **M.W. Laird** (2005) Use of the MET25 promoter system in *S. cerevisiae*. *Biotechnol Prog* 21:617-620.

8. **Laird, M.W.**, G.C. Sampey, K. Johnson, D. Zukauskas, J. Pierre, J.S. Hong, B.A. Cooksey, Y. Li, O. Galperina, J.D. Karwoski & R.N. Burke (2005) Optimization of BLyS Production in *Escherichia coli* K-12. *Protein Expr and Purif* 39:237-246.

9. Cooksey, B.A., G.C. Sampey, J. Pierre, X. Zhang, G.H. Choi, J.D. Karwoski & **M.W. Laird** (2004) *Bacillus anthracis* Edema Factor Production and Purification from *Escherichia coli* K-12. *Biotechnol Prog* 20:1651-1659.

10. **Laird, M.W.**, D. Zukauskas, K. Johnson, G.C. Sampey, H. Olsen, J.D. Karwoski, B.A. Cooksey, G.H. Choi, J. Askins, A. Tsai, J. Pierre & W. Gwinn (2004) Production and Purification of *Bacillus anthracis* Protective Antigen from *Escherichia coli* K-12. *Protein Expr and Purif* 38:145-152.

11. **Laird, M.W.**, K. Cope, R. Atkinson, M. Donahoe, K. Johnson & M. Melick (2004) Keratinocyte Growth Factor-2 Production in an *ompT*-Deficient *Escherichia coli* K-12 Mutant. *Biotechnol Prog* 20:44-50.

12. Innes, D., I.R. Beacham, C.A. Beven, M. Douglas, **M.W. Laird**, J.C. Joly & D.M. Burns (2001) The Cryptic *ushA* gene (*ushAc*) in Natural Isolates of *Salmonella enterica* (serotype Typhimurium) has been Inactivated by a Single Missense Mutation. *Microbiology* 147:1887-1896.

13. Raivio, T.L., **M.W. Laird**, J.C. Joly & T. Silhavy (2000) Tethering of CpxP to the Inner Membrane Prevents Spheroplast Induction of the Cpx Envelope Stress Response. *Mol Microbiol* 37:1186-1197.

14. Kloser, A.W., **M.W. Laird**, M. Deng & R. Misra (1998) Modulations in Lipid A and Phospholipid Biosynthesis Pathways Influence Outer Membrane Protein Assembly in *Escherichia coli* K-12. *Mol Microbiol* 27:1003-1008.

15. Kloser, A.W., **M.W. Laird** & R. Misra (1996) *asmB*, a Suppressor Locus of OmpF Assembly Mutants in *Escherichia coli*, is Allelic to *envA* (*lpxC*). *J Bacteriol* 178:5138-5143.

16. **Laird, M.W.**, A.W. Kloser & R. Misra (1994) Assembly of LamB and OmpF in Deep Rough Lipopolysaccharide Mutants in *Escherichia coli* K-12. *J Bacteriol* 176:2259-2264.

17. Edwards, R.A., E. Adderson, K.E. Klose, **M.W. Laird**, R.E. Lee, G.L. Miesel, F. Nano, R.M. Salerno & A. Hopkins (2008) Bacterial Select Agents and Antibiotic Resistance Genes. Manuscript submitted to *Emerg Infect Dis*.

Manuscripts in Preparation

1. Welch, C., S.S. Solow & **M.W. Laird** (2008) Development & Implementation of a Novel Plasmid Control for the Detection of Minute Virus of Mice (MVM) from Cell Culture. Manuscript in preparation.

2. X. Zhang, J. Askins, R. Fleming, B. Sturm, C. Poortman, P. Viriassov, B. Peterson, M. Flynn, Y. Miao, D. Zukauskas, R. Smith, G. H. Choi & **M.W. Laird** (2008) Selection of Potent Neutralizing Human Monoclonal Antibodies to Protective Antigen of *Bacillus anthracis*. Manuscript in preparation.

3. Solow, S.S., J. Sengbusch, A. Carbonello & **M.W. Laird** (2008) Protein production in *Saccharomyces cerevisiae* using alternative promoters. Manuscript in preparation.

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5. **Laird, M.W.**, K. Johnson, G.C. Sampey, D. Zukauskas, J.D. Karwoski, J. Askins, G.H. Choi & B.A. Cooksey (2008) Optimization of *Bacillus anthracis* Lethal Factor Production from *Escherichia coli* K-12. Manuscript in preparation.

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Patent Applications

1. Hewitt, D., Y.-H Kao, **M.W. Laird**, M. Schmidt & R. Wong (2007) Prevention of Disulfide Bond Reduction during Recombinant Protein Production of Polypeptides. Filed July 2007.

2. Rosen, C.A., A. Bell, P.A. Moore, Y. Shi, D.W. LaFleur, J.B. Bock, **M.W. Laird**, W.A. Haseltine, D.B. Woods & M. Subramanian (2006) Albumin Fusion Proteins. Patent Application No. 20080004206. Published 01/03/2008.

3. Yu, G.L, R. Ebner, J. Ni, C.A. Rosen, S. Ullrich & **M.W. Laird** (2002) Neutrokine Alpha & Neutrokine Alpha Splice Variants. Patent Application No. 20030175208. Published 09/18/2003.

4. **Laird, M.W.** (2001) Method of Making FGF-12 and KGF-2. Patent Application No. 20030119108. Published 06/26/2003.

5. Rosen, C.A., **M.W. Laird** & R.L. Gentz (2003) Antibodies Against Protective Antigen. Patent Application No. 10/602,702. Filed 01/25/2003.

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7. **Laird, M.W.** & D. Zukauskas (2001) Methods of Making Human Myeloid Progenitor Inhibitory Factor-1 (MPIF-1). Patent application No. 60/331,478. Filed 11/16/2001.

Recent Conference Session Chairing

(*upcoming*) Genentech Postdoc Offsite (October 2008) Aptos, CA

(*upcoming*) Biochemical Engineering Conference XVI (2009) Burlington, VT

(*upcoming*) RAFT VIII (2009) San Diego, CA

Co-chair for two sessions at the ACS National Meeting for the Upstream BIOT Division for Advances in Microbial Fermentation Process Development (2007) Boston, MA

Panel Member for CMC Strategy Meeting: Quality by Design (2007) Bethesda, MD

Co-chair at the 4th Recombinant Protein Production (RPP) Meeting: A Comparative View on Host Physiology (2006) Barcelona, Spain

Recent Conference Presentations

1. **Laird, M.W.** (2008) Recombinant Protein Production in *Escherichia coli*. Bacterial Genetics, Physiology & Biotechnology: Beckwith Reunion, Cassis, France. Abstract published.

2 **Laird, M.W.** (2008) Efforts to Understand and Control Glycosylation in CHO Cell Culture Processes. Cell Culture Engineering XI, Sunshine Coast, Queensland, Australia. Abstract published.

3. **Laird, M.W.** (2007) Recombinant Protein Production in *Escherichia coli*. Asilomar, Pacific Grove, CA. Abstract published.

4. **Laird, M.W.** (2007) Quality by Design in the Development of Post-Approval Changes. CMC Strategy Meeting: Quality by Design. Bethesda, MD. Abstract published.

5. **Laird, M.W.** (2006) Industry & Academia Relationship Round Table. 4th Recombinant Protein Production (RPP) Meeting: A Comparative View on Host Physiology (2006) Barcelona, Spain.

Honors and Awards

- Awarded $300 Travel Grant from the Arizona State Graduate College — 1995; 1996
- Department of Microbiology Alumni Scholarship Award of $500 — 1996
- Awarded a $200 Travel Grant for the *Best Oral Presentation* at the Arizona Branch of the American Society of Microbiology Conference — 1993
- Awarded Graduate Tuition Scholarship (GTS) — 1992-1996
- Awarded Graduate Academic Scholarship (GAS) — 1992-1993
- Iowa Centennial Scholarship Award for Academics — 1987-1991
- Iowa State University Academic Award of Excellence for Student Athletes — 1988-1991
- President's List for Student Athletes, Iowa State University — 1988-1991

- Central States Collegiate Hockey League (CSCHL) Player of the Week Honor Four Times — 1989-1990

Extracurricular Activities

- Member of the American Chemical Society — 2005-Present
- Appointed by Human Genome Sciences, Inc., President to the Employee Suggestion Review Committee — 2003-2005
- Poster Judge for Human Genome Sciences, Inc., Research Associate Poster Day — 2000-2005
- Founder & co-organizer of the *Advanced Bacterial Genetics Conference for Young Investigators* — 1999
- Committee member for David Innes, Ph.D. candidate; Griffith University, Australia. Chairman: Dr. Dennis Burns — 1998-1999
- Graduate Student Council Representative for Arizona State University — 1994-1995
- Columnist for the Arizona State University Graduate Voice Newsletter — 1994-1995
- State of Arizona Science Fair Judge — 1994-1996
- Member of the American Society for Microbiology — 1992-Present
- Writer for Genencor International, Inc., Bio-News Corporate Newsletter — 1991-1992
- Member of the Iowa State University Men's Division I Golf Team — 1986-1990
- Acting Captain of the Iowa State University Men's Division I Golf Team — 1988-1989
- Member of the Iowa State University Hockey Team — 1987-1991

Personal Summary

Highly self motivated and enthusiastic individual with excellent communication skills, decision making abilities, leadership capacities, and the aptness to work independently or as a member of a team. Thoroughly enjoys the challenges of life and scientific research & development.

EXHIBIT B

The Effects of Plasmid Content, Transcription Efficiency, and Translation Efficiency on the Productivity of a Cloned Gene Protein in *Escherichia coli*

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In order to investigate how plasmid content, transcription efficiency, and translation efficiency affect the productivity of a cloned gene protein, a new vector (pPLc-RP4.5) was constructed. The vector has P_L promoter, *lac*Z as a structural gene and 4.5S RNA gene between P_L promoter and *lac*Z gene. We took advantage of the characteristic that the 4.5S RNA is accumulated inside *E. coli* cells and can be quantitatively measured. A two-stage continuous culture system in combination with a temperature-sensitive gene switching system was used to study the performance of the recombinant fermentation. It was found that the plasmid content as varied by the dilution rate in the production stage showed a different pattern from that in the growth stage. The results showed that promoter strength had a greater influence on the overall gene expression efficiency of a cloned gene than the plasmid content, and the overall gene expression efficiency was largely dependent upon translation efficiency when a multicopy plasmid (pBR322 derivative and *rop*$^-$) and a strong promoter (P_L) were used to express a heterologous protein in *E. coli*.
Key words: P_L promoter • plasmid content • 4.5S RNA • gene expression • continuous culture, two-stage

INTRODUCTION

In spite of the fact that *Escherichia coli* has some drawbacks, such as its limited secretion capacity and lack of certain posttranslational modifications of proteins, which usually result in biologically inactive proteins, *E. coli* still remains a valuable host for the production of heterologous proteins because of its high growth rates, availability of low cultivation technology, and the advanced knowledge concerning its genetics and physiology.[3] A large variety of *E. coli* host–vector systems for the production of heterologous proteins are available, and sophisticated expression vectors have been developed to overcome difficulties encounted when overproduction is required.

Unstable plasmids could be stably maintained by inserting certain genes into vectors: genes for selection (antibiotics), survival function in combination with a host that requires the corresponding gene product for survival (Ssb, ValS),[26,35] or a partitioning system (ParA, ParB).[50,51] The lambda vector system is also used for stable cloned gene expression.[30] The use of strong promoters such as tac, trp, P_L, P_R, and T7 has led to increased final product yield, and inefficient translation initiation could be improved by using an efficient ribosome binding site, a specialized ribosome system or a two-cistron system.[14,20,29] The construction of fusion proteins,[22] secretion of overproduced proteins,[47] formation of inclusion body,[18] and introduction of *pin* gene[44] could help protect heterologous proteins from proteolysis as *lon*, *clpA*, *htpR* mutants do.[5,23,24]

In addition to the genetic factors mentioned above, the environmental factors influencing product formation should be studied to maximize the yield of a gene product from recombinant cells.[38] These factors cannot be studied separately when recombinant fermentation processes are to be optimized because they are highly interactive. Another important factor that affects the productivity of the protein encoded in a cloned gene is the physiology of the host cell. It has been shown that the same expression vector produced different amounts of proteins in a variety of host strains.[17] Although plasmid stability and product degradation often appear to be the determining factors, reasons for the change or difference in productivity remain obscure.[3]

In addition to the genetic characteristics of the host, an understanding of the relationship between the physiology of the host cell growth and the overproduction of a heterologous protein is of primary importance to the improvement of recombinant fermentation processes. When a cloned gene is expressed, the growth rates of recombinant cells are significantly reduced and sometimes these cells die.[31] An accumulation of the protein encoded in a cloned gene and an unbalanced state of the protein synthesizing system (i.e., the metabolic stress and/or the lack of protein synthesizing components for primary metabolism of host cells) might cause a decrease in the host cell growth rate and sometimes the death of host cells. These factors are closely related to the transcription and the translation of a cloned gene and its transcripts.

A two-stage continuous fermentation system in combination with a temperature-sensitive gene switching system has been used to study the performance of un-

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stable recombinant fermentation.[42] This system is able to separate the growth stage and the production stage simply by adjusting the culture temperature and to operate the production stage under well-defined experimental conditions.[19] The evaluation of the gene expression efficiency for a gene product was carried out by using a recombinant *E. coli* K12 ΔHΔtrp/pPLc23*trp*A1 as a host–vector model system in a two-stage continuous culture system.[19] Herein, we report the measurement of gene expression efficiency in terms of transcription and translation efficiencies by using the method developed by Brosius and Holy,[7] which was employed to measure the transcription efficiency of the *tpa* gene.[37] To apply this method to our recombinant fermentation system, we constructed a new plasmid. The newly constructed plasmid allowed us to simultaneously investigate the separate and independent effects of plasmid content, transcription efficiency and translation efficiency on the productivity of a cloned gene product. Through this approach, the individual effect of plasmid content, transcription efficiency and translation efficiency of the recombinant used was determined and the rate limiting step in recombinant fermentation processes was assessed.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Escherichia coli M72 (Sm, *lac*Zam, Δ*bio-uvr*, Δ*trp*EA2 [λ Nam7 am53, cI857 ΔH1])[4] was used as a host for the expression vectors and a source of the temperature-sensitive cI857 repressor. All the fermentation experiments were performed in Bioflo fermentors (New Brunswick Scientific, Edison, NJ). The seed culture was grown at 28°C in LB (1% tryptone, 1% NaCl, and 0.5% yeast extract) containing 100μg Ampicillin/mL.

When cultivated to early exponential phase, 10 mL of cell culture was used to inoculate the main cultivation vessel (working volume of 350 mL) under the conditions of 400 rpm and 1.0 L/min. For the two-stage continuous culture, the growth stage was changed to continuous mode of cultivation ($D = 0.5\ h^{-1}$) after initial 6–8 h period of batch culture. With the cells growing under steady-state conditions in the growth stage, the production stage (working volume of 350 mL, 400 rpm, 1.0 L/min) was inoculated, and continuous culture started.

The apparent specific growth rate in the production stage was controlled by adjusting flow rates, F_{12} and F_{02} (Fig. 1). The equation, $\mu_2^{app} = (D_{12} + D_{02}) - D_{12}(X_{1T}/X_{2T})$ was used to determine the apparent specific growth rate (where $D_{02} = F_{02}/V_2$, $D_{12} = F_{12}/V_2$, and X_{1T} and X_{2T} represent total cell mass in the growth stage and the production stage, respectively).[19] The growth medium was LB without ampicillin. Temperature was maintained at 28°C for the growth stage and at 40°C for the production stage.

Dilution rates in the production stage were changed after at least three residence times had elapsed and optical density remained constant. Changes of dilution rates were carried out in a way to empty the jar of the production stage after sampling, to refill the jar with new medium and cells from the growth stage, and to start a new culture at a different dilution rate just as the operation of the first dilution rate was performed. Viable cell counts were made on EMB agar plate and LB containing X-gal (40 μg/ml) and ampicillin (100 μg/ml).

Vector Construction

DNA manipulation was performed according to Maniatis et al.[21] pPLc23*trp*A1[36] was cleaved by EcoRI and PvuII, and the small fragment providing ampicillin-

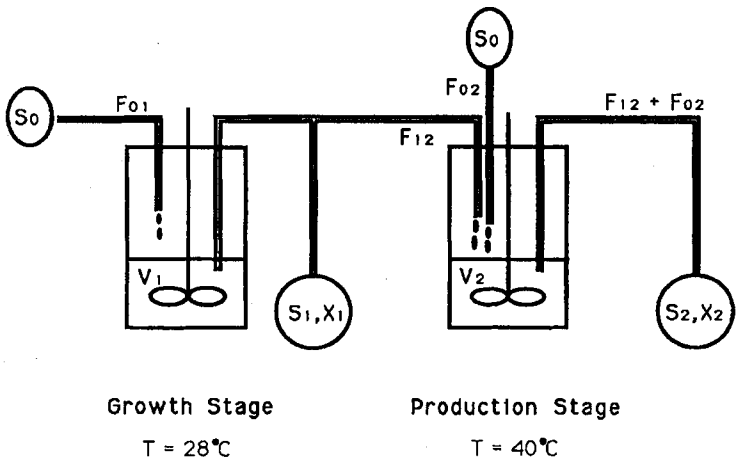

Figure 1. Schematic diagram of a two-stage continuous culture fermentation system; 1 and 2 indicate the first (growth, repressed) stage and the second (production, derepressed) stage, respectively.

resistance and PL promoter was ligated to the EcoRI–SnaBI fragment of pRS415[43] containing *lacZ*. The ligated plasmid (pPLc-RP) was used to transform the host, and was cut with EcoRI and BamHI after isolated from transformed cells. EcoRI-BamHI fragments containing 4.5S RNA gene from pKK290-2[7] was inserted into pPLc-RP cleaved by EcoRI and BamHI. The new plasmid, pPLc-RP4.5, construction (Fig. 2) was verified by restriction enzyme analyses.

Measurement of Plasmid Content

Plasmid DNA was isolated by the alkaline lysis method.[21] Gel electrophoresis was performed on 0.8–1.2% agarose in TAE buffer (40 m*M* tris–acetate and 1 m*M* EDTA, pH 8.0) with DNA linearized by EcoRI. The gel was stained in ethidium bromide (0.5 μg/mL) and recorded with Polaroid 55 negative film which was used for scanning (UltroScan XL) and estimation of the plasmid content. The linearized pBR322 by EcoRI was used as an internal standard for the plasmid content measurement.

Measurement of 4.5S RNA Content

For RNA isolation, a slightly modified method from ref. 10 was used. Cells resuspended in protoplasting buffer (15 m*M* tris–Cl, pH 8.0, 0.45*M* sucrose, 8 m*M* EDTA) were treated with lysozyme. Cells were lysed in 0.5 mL gram-lysing buffer (10 m*M* tris–Cl, pH 8.0, 10 m*M* NaCl, 1 m*M* Na-citrate, 1.5% SDS) containing proteinase K (100 μg/mL) and 15 μL of Diethylpyrocarbonate (DEPC). After adding 250 μL of 5*N* NaCl and centrifugating, the supernatant was collected. RNA was precipitated from the supernatant by adding ethanol. The pellet was washed in 70% ethanol, dried, and dissolved in 50 μL of double-distilled sterile water. Electrophoresis was carried out on 12% acrylamide, 0.4% bisacrylamide gel in 90 m*M* tris–borate buffer (pH 8.3). The gel was stained in ethidium bromide solution and recorded as described for the DNA gel.

Measurement of *lacZ* mRNA

The measurement of *lacZ* mRNA was performed by dot blot hybridization. Diluted RNA samples, which were

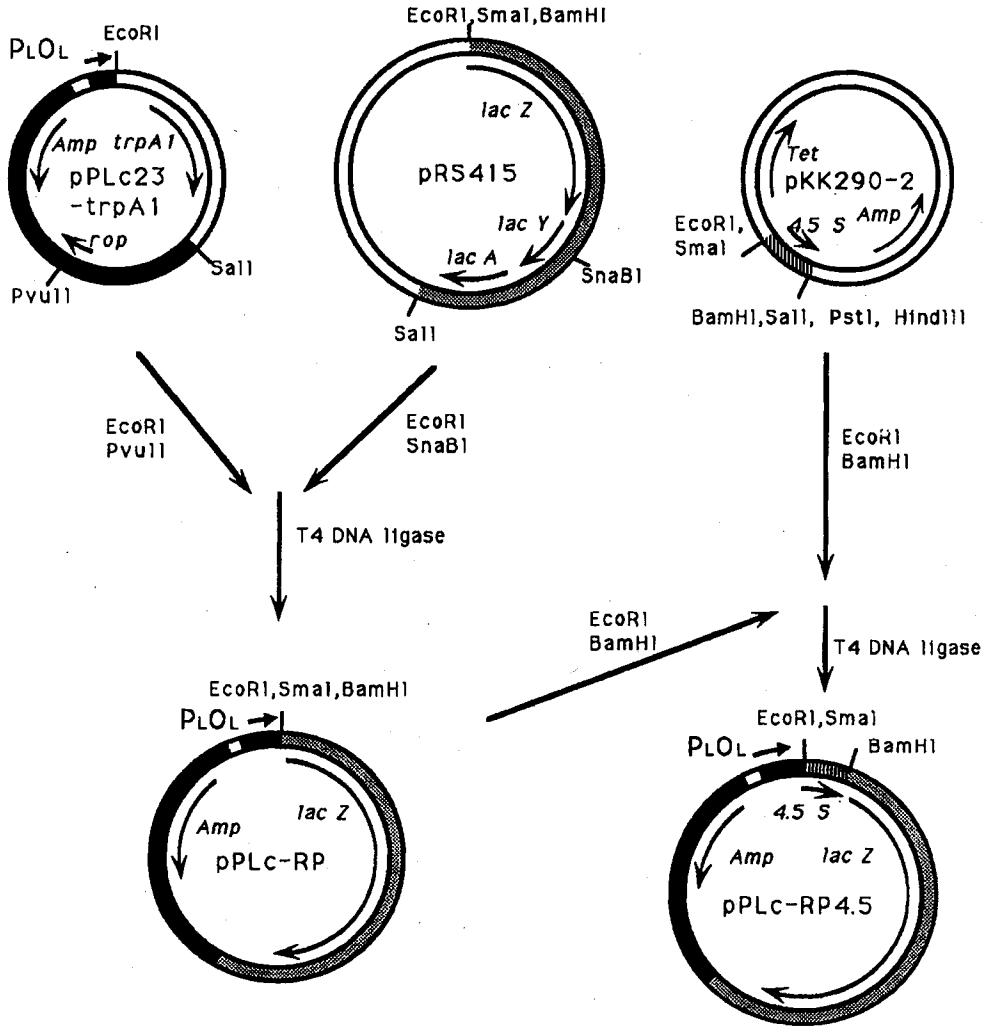

Figure 2. Schematic diagram of plasmid, pPLc–RP4.5, construction (Sizes are not to exact scale; only important restriction enzyme sites and structural genes are shown.)

used for 4.5S RNA measurements, were spotted onto a nylon membrane (Amersham) in 50 μL aliquots using filter apparatus (Biorad). The membrane was wetted in denaturing solution (1.5*M* NaCl, 0.5*M* NaOH) for 1 min and transferred to neutralizing solution (1.5*M* NaCl, 0.5*M* tris-Cl) for 1 min. The membrane was irradiated on a standard UV transilluminator (Spectroline model TR-302) for 5 min. Prehybridization was performed in a solution of 5 × SSC (diluted from 20 × SSC, 3*M* NaCl, and 0.3*M* sodium citrate, pH 7.0), 50% formamide, and 5% Denhart solution for 2 h at 42°C. Hybridization was carried out overnight at 42°C with large PvuII fragments of *lacZ* gene which were labelled with ^{32}P by random primer method (Amersham Kit).

Measurement of β-Galactosidase Activity

Assays of β-galactosidase were performed at 28°C according to Miller[25] following a chloroform and SDS treatment of samples withdrawn from the fermentation broth.

RESULTS AND DISCUSSION

Characteristics of pPLc–RP4.5 Plasmid

The plasmid pPLc–RP4.5 is carrying 4.5S RNA gene between PL promoter and *lacZ* gene. The 4.5S RNA is 114 nucleotide long, and normally accounts for 1–2% of the total small RNA in *E. coli*.[13] It was reported that 4.5S RNA is the only RNA present in ribonucleoprotein that resembles the mammalian signal recognition particle.[8] The RNA overproduction generally falls far short of the enhancement of gene copy number, but the overproduction of 4.5S RNA was found to parallel the increased gene dosage.[13] This unique characteristic correlation between the 4.5S RNA and the increased gene dose enabled us to make use of the newly constructed plasmids, pPLc–RP4.5.

Another important finding from the research is that the overproduced 4.5S RNA had no obvious effect on the cell growth rate.[13] So, the insertion of 4.5S RNA gene downstream of PL promoter and upstream of *lacZ* gene allowed us to measure transcription and translation efficiency independently of each other (Fig. 3).

In addition, pPLc–RP4.5 is expected to have more copy numbers than the original pPLc23*trp*A1 plasmid because the *rop* (*rom*) gene[48,49] was removed during the construction of pPLc–RP4.5. Nugent et al.[28] found that a plasmid that does not have the *rop* gene had 2–3 times more copy number than the original one. The PL promoter of the pPLc23*trp*A1 plasmid was reported to be completely repressed at 28°C by temperature sensitive repressors produced in the host cell.[36] However, the pPLc–RP4.5 plasmid is not under complete control so that it produces a small amount of β-galactosidase even at 28°C. That might be due to the lack of repressor

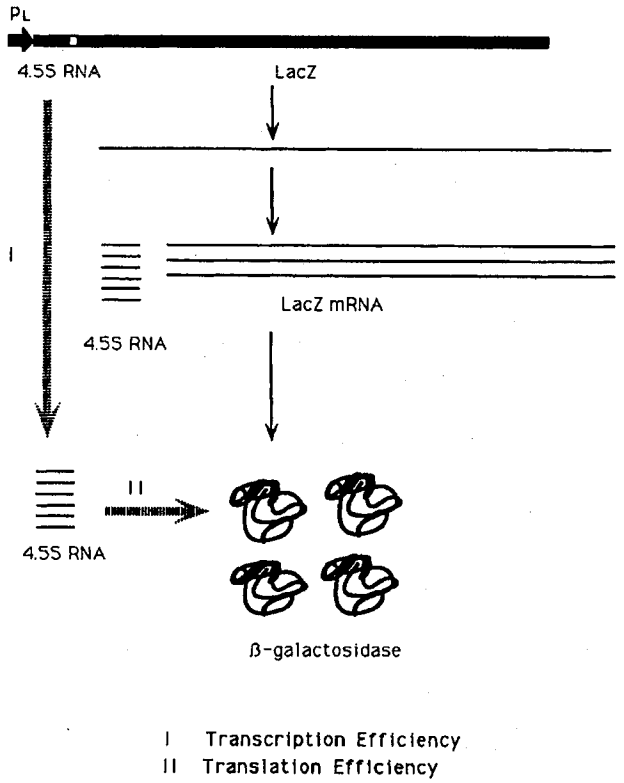

Figure 3. Schematic drawing for the measurement of the transcription efficiency and the translation efficiency independently of each other.

molecules to shut down the increased PL promoters. Because it is known that the copy number of the ColE1-like plasmid is not constant and either increases or decreases in a fashion that it is uncoupled from the specific growth rate,[40,42] continuous culture was carried out to see the effects of dilution rate on the plasmid contents and β-galactosidase activities.

As shown in Figure 4, β-galactosidase activities per hour decreased with the increasing dilution rate. The

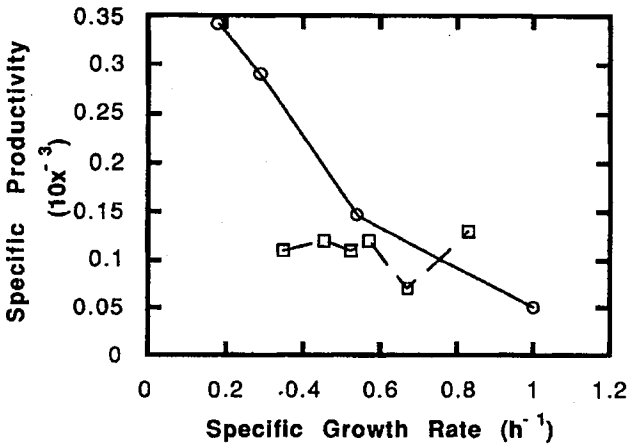

Figure 4. Specific productivity (Miller unit/h) of pPLc–RP4.5 in the growth stage at 28°C (-○-) and the host cell without pPLc–RP4.5 in the production stage at 40°C (-□-).

expression of the *lacZ* gene was almost controllable at $D = 1.0$. This fact suggests that the plasmid copy number decreased nearly to the number of active repressor molecules. And, very little β-galactosidase was produced when the nonrecombinant host cell was used (Fig. 4).

Figure 5 shows that the difference between the amount of pPLc23*trp*A1(42) and pPLc–RP4.5 was narrowed as the dilution rate decreased. This data implies that there might be some functional relationship between *rop* gene product and plasmid replication depending on the specific growth rate.

Intracellular 4.5S RNA and *lacZ* mRNA Level

The intracellular 4.5S RNA accumulated during fermentation was measured by scanning the negative film with laser densitometer after being separated on polyacrylamide gel and stained with EtBr. Cells grown in the repressed condition have accumulated very little 4.5S RNA and cells grown in the derepressed condition have different amounts of 4.5S RNA depending on the various dilution rates (Fig. 6). Scanned 4.5S RNA bands were corrected for differences in cell density, gel background, and any experimental errors by comparing total RNA and total small RNA of each sample. Since the 4.5S RNA gene is located upstream of *lacZ* gene and start of transcription does not always guarantee production of full length mRNA, the transcription of the 4.5S RNA gene does not necessarily mean that functionally active β-galactosidase mRNA is made. A tight coupling between transcription and translation is a general phenomenon in prokaryotes. Thus, a reduced translation will increase the probability that the RNA polymerase comes off from the template before it reaches the 3′-end of the gene. This polar effect was also observed by Rothstein et al.,[37] who measured the amount of 4.5S RNA that was inserted at various positions within the *tpa* gene.

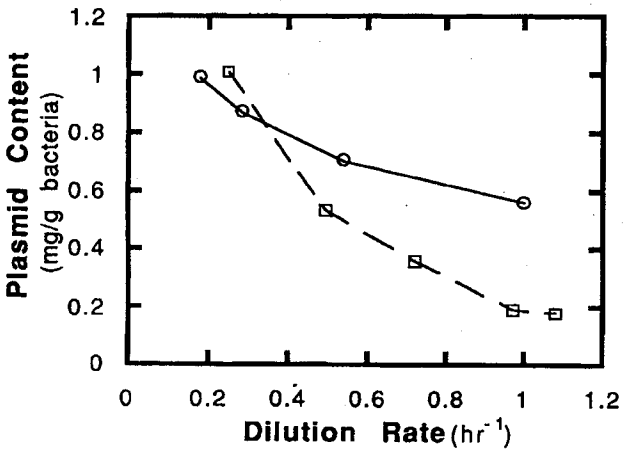

Figure 5. The effect of dilution rate on the plasmid content (mg DNA/mg total cell) in the growth stage: pPLc–RP4.5 [*rop* $^-$ (-○-)] and pPLc23*trp*A1 [*rop* $^+$, (-□-)] (from ref. 19).

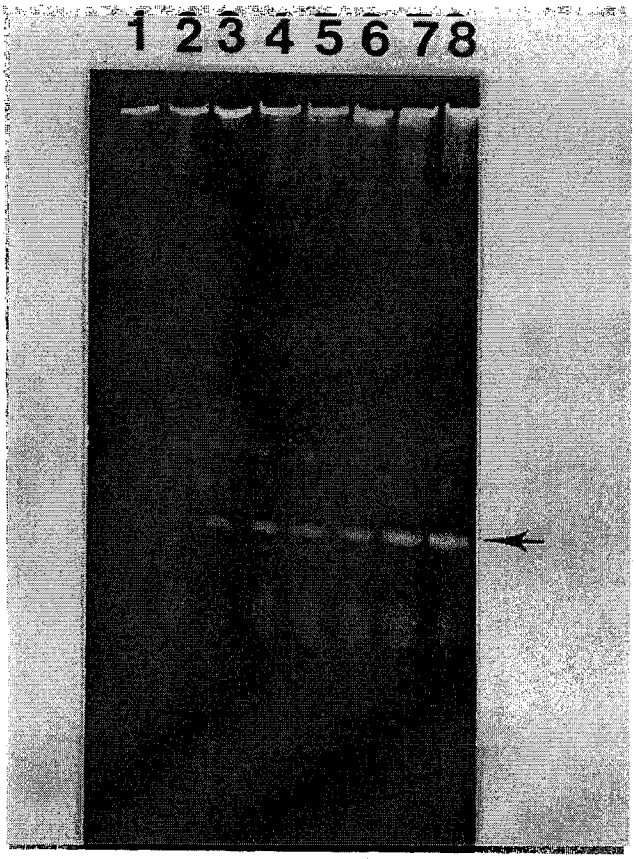

Figure 6. Expression of 4.5S RNA with varying dilution rates. Lanes 1 and 2 show $D = 0.5\ h^{-1}$ in the growth stage; lane 3 shows $D_2 = 0.74\ h^{-1}$; Lane 4 shows $D_2 = 0.59\ h^{-1}$; lane 5 shows $D_2 = 0.47\ h^{-1}$; lane 6 shows $D_2 = 0.43\ h^{-1}$; lane 7 shows $D_2 = 0.27\ h^{-1}$; lane 8 shows $D_2 = 0.19\ h^{-1}$, all in the production stage. The arrow indicates 4.5S RNA.

Stassens et al.[45] also found that translation efficiency controlled the mRNA level when they measured the steady state level of *lacZ* mRNA and β-galactosidase activity. They suggested that prematurely terminated *lacZ* mRNAs, which were rapidly broken down, were generated by the transcriptional polarity which can be caused by an increased spacing between the RNA polymerase and the leading ribosome.

We assume that RNA polymerase generally completes each transcription event when new mRNAs coming up from transcription complex are followed by translation. In other words, the completion of transcription depends not only on transcription complex itself, but on translation that prevents RNA polymerase from coming off from the DNA template. When these factors are taken into consideration, the measurement of *lacZ* mRNA using ^{32}P-labelled *lacZ* gene fragment may not give us an accurate transcription efficiency.

Figure 7 shows the comparison between the amount of 4.5S RNA accumulated and the steady-state level of *lacZ* mRNA. We found that the difference between the amount of 4.5S RNA in the growth stage (repressed condition, lane 1) and those in the production stage (derepressed condition, lanes 2–6) is greater than the

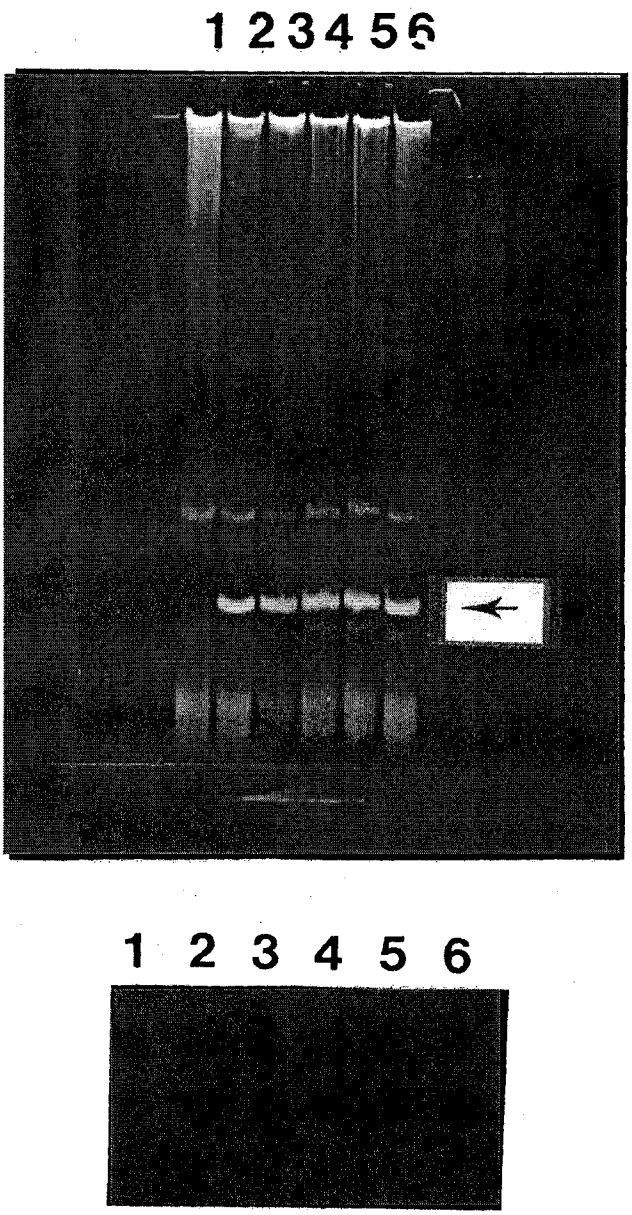

Figure 7. Comparison between polyacrylamide gel electrophoresis of 4.5S RNA (10 μL total RNA sample) and dot blot hybridization of *lac*Z mRNA (5 and 2.5 μL total RNA sample). The lane numbers on both plates indicate the corresponding sample number. The specific productivities (Miller unit/h) shown in lanes 1, 2, 3, 4, 5, and 6 are 0.35×10^3, 11.8×10^3, 15.7×10^3, 9.5×10^3, 9.0×10^3, and 7.7×10^3, respectively. The arrow indicates 4.5S RNA.

difference between the amount of *lac*Z mRNA accumulated in the growth stage (No. 1) and those in the production stage (Nos. 2–6). Because the specific β-galactosidase activity (0.35×10^3) of lane 1 (or No. 1) is at least 20 times lower than the lowest one (7.7×10^3) among lanes 2–6 (or Nos. 2–6), it is evident that measuring the accumulated 4.5S RNAs is more reliable than measuring the steady state *lac*Z mRNA level for a quantitative analysis of the transcription efficiency of the given gene with a strong promoter (P_L). Therefore, the analytical method used for determination of the accumulated 4.5S RNA enables us to measure the transcription efficiency with accuracy, ease, and rapidity.

Transcription and Translation Efficiency

The maximum productivity of a cloned gene protein is generally thought to be related to plasmid copy number (gene dosage effect),[2] transcription efficiency (strength of promoter),[1] translation efficiency (efficient binding of ribosomes to translation initiation sites, which is affected by the Shine–Dalgarno sequence, the codon usage and the secondary structure of mRNA),[12,39,41] the stability of mRNA (functional half life of mRNA),[34] and the stability of a cloned gene product itself.[33] All of these factors are not independent, but highly interactive, and should be properly balanced in order to obtain maximum productivity. For instance, a cloned gene with a weak and constitutive promoter will be greatly influenced by plasmid copy number,[33] but use of a strong promoter is going to reduce the gene dosage effect by increasing transcription efficiency.[1]

We investigated the relationship between plasmid content, transcription efficiency and translation efficiency by using the newly constructed pPLc–RP4.5 plasmid which has P_L promoter, 4.5 RNA gene, and *lac*Z gene. Figure 8 shows the specific productivity of β-galactosidase and the plasmid content as a function of specific growth rate in the production stage. Although plasmid content decreased as the dilution rate (specific growth rate) increased in the growth stage (Fig. 5), plasmid content remained almost constant in the production stage. The following explanations may be offered.

Stueber et al.[46] found that transcriptional readthrough over plasmid replication origin from the opposite direction caused a decrease in plasmid copy number. So, active transcription from P_L promoter in the production stage seemed to have some effects on plasmid replication. Another possible reason comes from the opera-

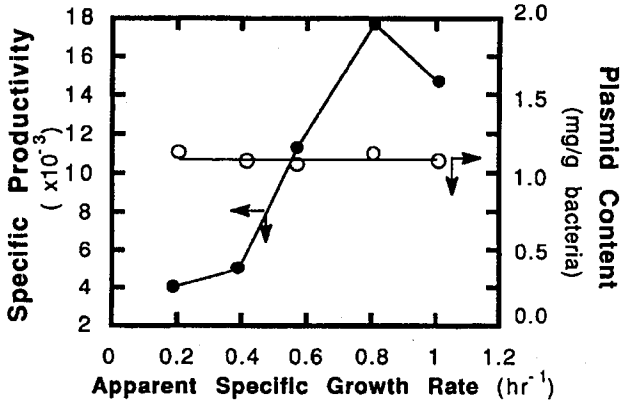

Figure 8. The effect of specific growth rate on the plasmid content and the specific productivity in the production stage: (-●-) specific productivity in Miller unit per hour; (-O-) plasmid content, mg/g total cell.

tional characteristics of a two-stage continuous culture system. New cells continuously come into the production stage from the growth stage, and the apparent specific growth rate is a bulk parameter representing the growth rate of heterogeneous plasmid-harboring and plasmid-free cell population. Actually, plasmid-harboring cells, when they are actively expressing cloned genes, grow slower compared to plasmid-free cells. Therefore, depending on the operational method of adjusting dilution rate and apparent specific growth rate in the production stage, there might be some variations in plasmid content.

Figure 8 shows that specific productivity has changed as the apparent specific growth rate changed despite the constant plasmid content. This fact indicates that specific productivity has little to do with plasmid content in the expression stage of this recombinant fermentation system. It should be noted that the fraction of plasmid harboring cells apparently varies as the specific growth rate changes in the production stage. For example, at specific growth rates of 0.1 and 0.5 h^{-1}, the fractions of plasmid harboring cells were 0.65 and 0.3, respectively, when the fraction of plasmid harboring cells in the growth stage was 0.8.[32] Corrections should be made to measure the plasmid content per gram of plasmid-harboring cells and the specific productivity of plasmid harboring cells. The plasmid content and the specific productivity per gram of total bacteria, however, are used here.

When recombinant cells carrying plasmids begin to express a cloned gene product by derepressing the P_L promoter, they become large and morphologically different from plasmid-free cells.[31] It was observed that a large number of plasmid-harboring cells lost their colony-forming ability when plated on agar surfaces. Most of the non-colony forming cells are viable and keep producing the gene product in the bioreactor.[31]

Considering these observations (size differences, non-colony-forming cells), we assume that the fraction of plasmid harboring cells in terms of mass (both colony forming and non-colony forming cells) would be almost constant although the fractions in the total colony forming unit of plasmid-harboring cells are different. The replication of only one plasmid at a time in the ColE1-like plasmids requires long generation time (slow growth rate) to obtain high plasid copy number or content. The division of plasmid content by the plasmid harboring cell fraction, however, results in an increased plasmid content at a higher specific growth rate (Table I), which is contradictory to the replication mechanism of the ColE1-like plasmids. Also, we may be able to explain why the fraction of plasmid-harboring cells decreases as μ_2^{app} increases with the following simple equations.

$$dX/dt = \mu X \quad (1)$$

$$\ln(X/X_0) = \mu t \quad (2)$$

By putting $X = 2X_0$ and $t = t_d$ into eq. (2), we obtain

$$t_d = 0.693/\mu \quad (3)$$

where t_d is generation time and X_0 is biomass at $t = 0$. This equation shows that as μ decreases, t_d drastically increases. However, when the second stage is operated under the condition of a fixed dilution rate ($D_2 = 0.7$ in ref. 32, $D_2 = 1.0$ in this article), we have the same residence time through all the values of μ evaluated. Plasmid-harboring cells that are actively expressing heterologous proteins seem to be somewhat less reproductive, namely, defective of machinery for cell division. This suggests that the number of divisions of plasmid-harboring cells at high growth rates would be little different from that at low growth rates even though cell mass increases faster at higher specific growth rates. The plasmid-free cells, however, will have more chances to divide themselves at a higher specific growth rate than at a low specific growth rate at a given mean residence time in the bioreactor. Therefore, more cell divisions of the plasmid-free cells than those of the plasmid-harboring cells at a high specific growth rate may give rise to the difference in the fraction of plasmid-harboring cells, although the change in the mass ratio of the plasmid-free cells to the plasmid-harboring cells are small enough to be ignored.

Due to the design and operating conditions of a two-stage culture system, the dilution rate in the production stage is always greater than the specific growth rate [$\mu_2 = D_2 - D_{12}(X_1/X_2)$]. The second-stage jar is emptied and refilled with new cells and medium after each sampling and adjustment of dilution rate. For these reasons, plasmid-free cells do not have enough time to outgrow plasmid-harboring cells in the production stage.

We measured transcription and translation efficiency by analyzing plasmid content, 4.5S RNA content, and specific productivity to see which was responsible for the increase or decrease of the specific productivity as the specific growth rate changed when multicopy

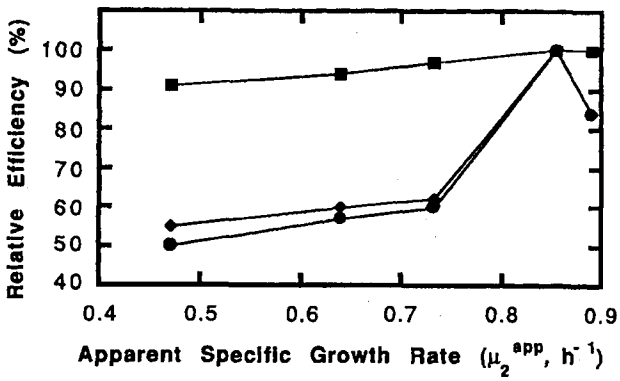

Figure 9. The effects of apparent specific growth rate on the overall gene expression efficiency (-●-), transcription efficiency (-■-), and translation efficiency (-◆-).

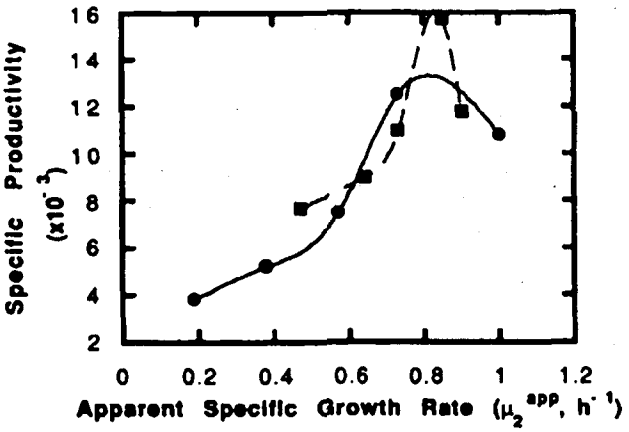

Figure 10. The effect of apparent specific growth rate on the specific productivity (Miller unit per hour): (-●-) constant $D_{12} = 0.15\ h^{-1}$; (-■-) constant D_2 ($D_{12} + D_{02}$) $= 1.0\ h^{-1}$.

plasmid and strong promoter were used to express a cloned gene product (Table I). Transcription efficiency (4.5S RNA/μg DNA · h) was calculated by dividing the amount of 4.5S RNA transcribed per unit time by the amount of plasmid. Since absolute values of 4.5S RNA content could not be obtained, efficiencies relative to the highest efficiency (100%) among samples tested were measured. Calculation of translation efficiency (β-galactosidase/4.5S RNA · h) was done in the same way. Overall gene expression efficiency (β-galactosidase/μgDNA · h) is obtained by dividing the amount of β-galactosidase produced per unit time by the amount of plasmid.

Figure 9 shows that transcription efficiency remains almost constant within the range of the specific growth rates evaluated, but translation efficiency changes significantly depending on the specific growth rates. It appears that RNA polymerase produces mRNA at almost the same rate within the given range of the specific growth rates tested when strong P_L promoters on multicopy plasmids are its targets, but the capacity of the protein synthesizing system cannot keep up with transcription efficiency, especially at low specific growth rates.

It was found that (1) individual mRNA chains, on average, are translated more times in rapidly growing cells having a higher concentration of ribosomes than in slowly growing cells having less ribosomes,[6] (2) the average ribosome efficiency decreases significantly at low specific growth rates,[11] and (3) the rate of total protein synthesis correlated better with the number of ribosomes than with the amount of total mRNA.[27] The increased specific growth rate augments cellular components involved in the protein synthesizing system that comprises not only the ribosomes, tRNA, and mRNA, but a whole set of indispensible proteins such as aminoacyl-tRNA synthetases.[15] Therefore, it is believed that as more of cellular components required for the protein synthesis are available the translation efficiency increases, so that the increased specific productivity is observed at a higher specific growth rate.

A similar pattern of correlation between productivity and apparent specific growth rate is exhibited by two different operational methods [constant $D_{12} = 0.15\ h^{-1}$ and constant $D_2(D_{12} + D_{02}) = 1.0\ h^{-1}$] (Fig. 10). A drop in the specific productivity at $\mu_2^{app} = 0.9$ may be due to the maximum growth rate of plasmid-harboring cells in the given environment. The apparent growth rate can increase over the maximum growth rate of plasmid-harboring cells in the second stage because there is no washout dilution rate in the second stage of a two-stage continuous culture, and plasmid-free cells contribute to the increase in the apparent growth rate.

It was reported that an increased specific productivity of consensus interferon was obtained with fast growing cells,[9] and Jensen and Carlsen[16] argued that the lack of dependence of the specific production rate on the specific growth rate in their system used could be due to the efficient translation system. From all the data presented here and the literature references mentioned above, we may draw a conclusion that the production of a heterologous protein in *E. coli* is at least partially dependent on the growth rate, and an increased productivity can be achieved by increasing the growth rate up to a certain point which corresponds to a maximum productivity. Further studies, however, are needed to ascertain the more general applicability of these results for other recombinant fermentation systems.

CONCLUSIONS

The new plasmid, pPLc-RP4.5, which contains the 4.5S RNA gene between P_L promoter and *lac*Z gene, was used to evaluate gene expression efficiency in

Table I. Measurement of gene expression efficiency.

μ_2^{app} (h^{-1})	DNA[a] (μg)	4.5S RNA[b] (arbitrary unit)	β-gal/h ($\times 10^{-3}$)	Relative[c] transcription efficiency(%)	Relative[c] translation efficiency(%)	Overall gene[c] expression efficiency(%)	Plasmid-harboring cell fraction
0.48	0.85	35	7.69	91	56	53	0.39
0.64	0.91	38.4	9.07	93	60	56	0.31
0.73	0.9	39	9.52	95	62	59	0.33
0.85	0.88	40	15.77	100	100	100	0.30
0.9	0.78	35.5	11.8	100	84	84	0.25

[a] The same amount of cell mass (equivalent to O. $D_{400} = 2.0$) was analyzed.
[b] Relative amount of 4.5S RNA was determined in arbitrary unit as % area of the total scanned area.
[c] To facilitate comparisons showing efficiencies, the efficiencies of the sample from $\mu_2^{app} = 0.85$ were arbitrarily set at 100%.

terms of plasmid content, transcription efficiency, and translation efficiency. It was found that the specific productivity varied independently of plasmid content in the production stage and that the augmented translation efficiency caused by increased specific growth rate might play a major role in increasing the productivity of a cloned gene product.

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EXHIBIT C

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Enhancing recombinant protein yields in *Escherichia coli* using the T7 system under the control of heat inducible λP_L promoter

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Abstract

A recombinant plasmid containing the complete *lacZ* gene downstream of the T7 promoter was used to transform *Escherichia coli* containing another plasmid which had the T7 RNA polymerase gene under the control of heat inducible λP_L promoter. This recombinant *E. coli* containing the two plasmids was studied in order to enhance β-galactosidase expression. The heat shock time which effectively regulates the T7 RNA polymerase was optimized and best expression of β-galactosidase was obtained with 2 min heat shock. Substrate feeding increased the duration of log phase and allowed induction at a higher cell density without affecting the specific activity. A high cell density (7 g l^{-1}) and high specific activity ($\sim$ 20 000 U) were achieved which effectively increased the product concentration 18-fold.

Keywords: Recombinant; *E. coli*; Overexpression; β-galactosidase; T7 promoter; λP_L promoter; Heat shock; Fed batch

1. Introduction

The T7 system comprising the T7 promoter and T7 RNA polymerase is one of the most commonly used systems for recombinant protein overexpression in *Escherichia coli* (Tabor and Richardson, 1985; Studier and Moffait, 1986). However, though the specific activities are high (more than 50% of cellular protein) (Studier et al., 1990), the biomass yields are poor leading to low protein yields per ml of culture.

The availability of nutrients especially during the phase when the cells are fully induced is often critical for recombinant protein yield (Yee and Blanch, 1993a). Thus, even for recombinant proteins expressed under the control of strong promoters, production is growth associated and a high rate of protein production is achieved only when the cells are in the exponential phase (Kim

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and Ryu, 1991). There are structural models which link recombinant protein production to total RNA levels, which again is a growth associated function (Gourse et al., 1985).

Attempts to increase the product concentration (protein per ml of culture) by either delaying the induction time or harvesting after a longer period post induction has a deleterious effect on the specific activity offsetting any gain achieved by the increase in cell biomass. This is possibly due to nutrient starvation in the culture which leads to a lowering of specific growth rates and hence a concomitant lowering of rates of foreign protein production.

The need for a costly inducer like IPTG in the T7 system where the T7 RNA polymerase gene is under the control of *lac* promoter (e.g. *E. coli* BL21 (λDE3)) increases the fermentation cost and is thus not preferred in scale-up. In addition, for recombinant proteins of therapeutic/pharmaceutical importance the presence of IPTG further complicates downstream processing. Considering this, the use of the heat-inducible λP_L promoter to induce T7 RNA polymerase synthesis in the T7 system is more preferable.

We therefore chose a model system where the T7 RNA polymerase gene is under control of λP_L promoter which can be induced by heat shock at 42°C. However, the appropriate conditions for optimum induction of the λP_L promoter are not clearly addressed in literature. Some authors suggest increasing temperature from 30 to 42°C for 15 min, then reducing temperature to 37°C to induce transcription (Hui et al., 1987), while others recommend increasing the temperature to 42°C for the remainder of the experiment after a suitable density has been obtained (Remaut et al., 1981, 1983; Shatzmant and Rosenberg, 1986). Also there are reports, which are product specific on the optimum duration, as well as the temperature at which heat shock should be given (Okita et al., 1989).

This paper attempts to enhance recombinant protein production by controlling the synthesis of T7 RNA polymerase by optimizing the heat shock strategy. The intermittent substrate feeding strategies are also designed to increase the cell density without compromising the specific activity. The choice of *lacZ* as the reporter gene was dictated by the ease and linearity of the assay for β-galactosidase.

2. Materials and methods

2.1. Strains, plasmids and expression vectors

The T7 based expression vectors pRSET A, B, C (Invitrogen) (amp®) were used for cloning the reporter gene. The plasmid pGP1-2 (kanamycin®) is a derivative of pACYC177 and was used for expression of T7 RNA polymerase under the heat inducible λP_L promoter (Tabor and Richardson, 1985). The complete 3-kb *lacZ* was taken out from plasmid pCH110 (Pharmacia Biotech) (amp®). The plasmids were maintained and propagated in *E. coli* DH5α (recA1, thy1, hsd17, snpE44, gyrA96).

2.2. Growth media and cultivation conditions

Bacterial cells were routinely grown at 37°C in LB medium (10 g l^{-1} bactotryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl) at 200 rpm. Ampicillin (100 mg l^{-1}) and kanamycin (50 mg l^{-1}) were added when required to maintain selection pressure. Modified LB medium consisted of LB medium supplemented with 5 g l^{-1} K_2HPO_4 in which 10 mM $MgSO_4$, 1 ml l^{-1} trace metal solution (Shiloach and Baner, 1975) and glucose at a concentration of 0.25% were added post autoclaving. Concentrated medium (5 ×), which was used for feeding, contained all the above constituents at 5 × concentration other than K_2HPO_4, which was added at 1 × concentration. Glucose concentration was increased to 0.5% for bioreactor studies. Media were solidified by the addition of 1.5% agar.

The fermentation was conducted in 100 and 500-ml Erlenmeyer flasks for 10 and 50 ml cultures respectively at 200 rpm. Bioreactor studies were performed in a computer controlled 3-l bioreactor (Bioflo 2000 Fermentor, New Brunswick Scientific). Water circulators at 45 and 5°C were used for heating and cooling, respectively.

2.3. Recombinant DNA techniques

Standard DNA techniques (Sambrook et al., 1989) were used for construction of the recombinant plasmid. Enzymes were purchased from different manufacturers and were used as recommended. Wizard purification systems (Promega Corporation) were used in small-scale and large-scale plasmid isolation and purification of DNA fragments from agarose gel. Standard $CaCl_2$ method was used to transform *E. coli*.

2.4. Cell density measurement

Cell density was measured by dry weight (X_T) and optical density (OD) methods. For determining dry weight, 5 ml of the sample was spun in a preweighed centrifuge tube at $8000 \times g$ for 5 min. The cell pellet was washed with deionized water and dried at 100°C for 24 h. The tube was weighed along with the dried pellet and used to calculate the dry weight of the sample.

The OD was measured by Hitachi model U2000 spectrophotometer at 600 nm. The culture was appropriately diluted to read OD_{600} in the linear range of measurement (0.05–0.50). A standard plot was constructed between dry cell weight and OD. The correlation between them was determined as $X_T = (0.5 \cdot OD_{600})$ g l^{-1}.

2.5. β-Galactosidase assay and total protein

Blue-white screening with Xgal-IPTG was used to select the desired recombinant cell on plate. In liquid culture, the enzyme was assayed by ONPG test on permeabilized *E. coli* cells according to Miller (1972). The culture was appropriately diluted to maintain the linearity of the assay and prevent substrate limitation. The specific activity is expressed in terms of Miller units (U). The final product concentration is measured in terms of volumetric activity, which is defined as the product of specific activity and OD_{600} of the culture. Thus the unit of product concentration is U ml^{-1}. All assays were carried out in triplicate.

Total protein was estimated by assuming that the *E. coli* culture has a total protein content of 150 μg ml^{-1} at a cell concentration of 10^9 ml^{-1} (Miller, 1972).

2.6. Administering 'heat shock'

Cells were initially cultured in a shake flask at 30°C. For heat shock, the temperature was raised to 42°C by transferring the flask to a shaker water bath at 43°C. This allowed the temperature of the medium to rise to 42°C in ~2 min. Effective heat shock time was calculated after this temperature was achieved. After heat shock, the flask was shifted to another shaker water bath at 35°C that allowed the temperature to drop to 37°C in 2 min.

3. Results and discussion

3.1. Construction of lacZ-T7-promoter fusion

The 4-kb *Kpn*I–*Pst*I fragment containing the complete *lacZ* gene from plasmid pCH110 was inserted into the *Kpn*I–*Pst*I digested plasmid pUC19 that resulted in plasmid pUC-lacZ (6.7 kb). This plasmid was digested with *Hin*dIII complete and *Eco*RI partial (since the *Eco*RI site is also present in the *lacZ* gene) to get a 4-kb fragment which was ligated to the *Eco*RI–*Hin*dIII digested pRSET A, B and C. This resulted in cloning of the *lacZ* gene in all three reading frames under the T7 promoter. The pRSET B-lacZ (Fig. 1) gave maximum activity indicating the insertion of the gene in correct reading frame in this plasmid.

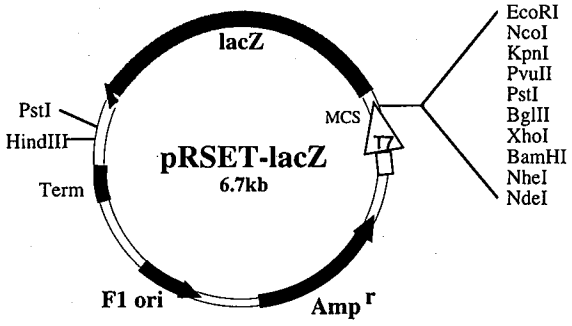

Fig. 1. Schematic of recombinant plasmid containing the gene for β-galactosidase under the T7 promoter.

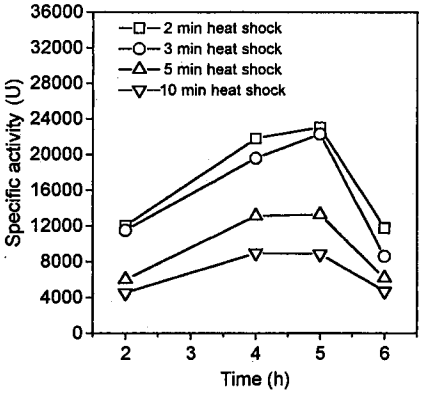

Fig. 2. Time profile of β-galactosidase expression with different durations of heat shock.

3.2. Optimizing the 'heat shock' time

It is well known that prolonged induction tends to disrupt the cellular machinery leading to poor specific growth rates (Lee and Ramirez, 1992). In addition, it is well established that the expression of the recombinant protein under the control of the T7 promoter is highest when the expression of the T7 RNA polymerase is suboptimal. This is primarily because the T7 RNA polymerase has a high transcriptional efficiency and only a small amount is required for high expression (Chamberlin et al., 1970). Usually the rate-limiting step for protein synthesis does not lie at the transcriptional level with the T7 based expression systems. Higher levels of T7 RNA polymerase seem to be 'toxic' to the cell (Tabor and Richardson, 1985) and serve no useful purpose while diverting the essential metabolites away from recombinant protein production. Since T7 RNA polymerase expression is directly related to the time for which heat shock is given it was decided to determine the optimum duration of heat shock.

Cells were grown at 30°C thereby completely repressing the λP_L promoter, in LB medium until the cell density reached 0.3 g l^{-1}. Heat shock are given at this point for different time durations at 42°C to differentially induce the λP_L promoter. Activity and cell density were monitored over time and maximum activity was observed approximately 4–5 h post heat shock irrespective of the duration for which heat hock was given (Fig. 2). It was observed that the cultures induced for 2 and 3 min gave higher specific activities as compared to 5 and 10 min induced cultures. This was attributed to an optimal level of induction of λP_L promoter because all the cultures started with negligible baseline activity.

The cell density, specific activity and product concentration achieved 4 h after heat shock was given in Fig. 3. It was observed that even when cells were given no heat shock, i.e. temperature was simply shifted from 30 to 37°C, a fairly high specific activity of 11 000 U was achieved 4 h after the shift up. This indicated that the T7 RNA polymerase got expressed at 37°C, albeit at lower levels. The specific growth rate fell sharply when heat shock was given even for 1 min, thereby demonstrating that growth almost ceases the moment cells are induced. This is similar to the observation made by other authors, that when recombinant organisms containing strong promoters, are induced, the specific growth rate declines sharply (Seo and Bailey, 1985). A sharp peak in maximum activity was observed when heat shock was given for just 2 min. This was about 6-fold the activity measured with 15 min of 'heat shock' which is the time required for maximal induction of the λP_L promoter. Thus, a very low and controlled level of expression of T7 RNA polymerase is required for best expression of recombinant protein. It was therefore decided to

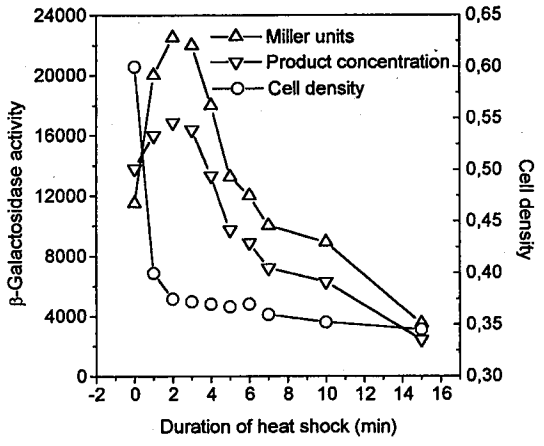

Fig. 3. Effect of duration of heat shock on cell density (g l^{-1}), specific activity (U) and product concentration (U ml^{-1}).

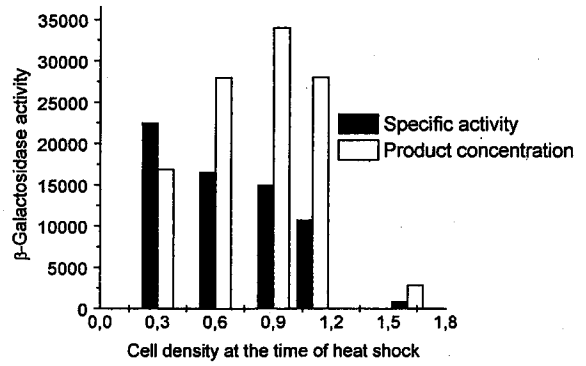

Fig. 4. Specific activities (U) and product concentrations (U ml^{-1}) measured 4 h post heat shock.

keep the heat shock time constant at 2 min for all further experiments.

3.3. Effect of nutrient availability

In an attempt to increase the final product concentration, heat shock was given at higher cell densities. However, it was observed that specific activity declined sharply from 22 500 U when heat shock was given at $X_T = 0.3$ g l^{-1} to 680 U when heat shock was given at $X_T = 1.6$ g l^{-1} (Fig. 4). To investigate whether this was due to nutrient limitation, the cells were pelleted after growth to a comparatively higher cell density ($X_T = 1.45$ g l^{-1}) and then resuspended in fresh medium. After shaking for about 30 min at 30°C, heat shock was given for 2 min when cell density was 1.75 g l^{-1}. An increased specific activity of 24 875 U was observed as compared to the control (890 U) where no pelleting followed by resuspension in fresh medium was done. This demonstrated that nutrient availability was critical for high product yields (Table 1).

The possibility of any kind of byproduct accumulation that might reduce cell growth leading to poor activity at higher cell densities was also investigated. The culture was grown to high cell densities and then fresh nutrients were supplied by adding, one-fifth of culture volume, the concentrated 5 × medium. The byproduct, if present would not be critically diluted and thus no increase in activity should be observed. However, this experiment led to a sharp increase in specific activity from 890 to 23 760 U on addition of fresh nutrient (Table 2). This demonstrated that the poor activity observed in the control was essentially due to nutrient limitation in the culture.

3.4. Fed batch strategy

Since the supply of nutrients was critical to good expression, it was decided to adopt a fed batch strategy for improving recombinant protein concentration in the culture yields. For this, the recombinant cells were cultured in 50 ml LB modified medium and the growth profile was monitored. It was observed that the specific growth rate started declining when the cell concentration (X_T) reached ~1.5 g l^{-1}. Therefore, 10 ml of concentrated medium (5 ×) was added at this time point following which the heat shock given for 2 min at $X_T \sim 1.5$ g l^{-1}.

The maximum product concentration (75 000 U ml^{-1}) was observed 8 h after heat shock which corresponded to a specific activity of 16 500 U at a cell density of 2.25 g l^{-1} (Fig. 5A). Since the specific activity was comparatively low, it was decided to give nutrient feed even after heat shock. Two strategies of either giving one or two feeds were adopted. The results showed that giv-

Table 1
Effect of resuspending cells in fresh medium on β-galactosidase expression

Cells resuspended at (X_T) [g l^{-1}]	Heat shock given at (X_T) [g l^{-1}]	X_T after 4 h induction [g l^{-1}]	Specific activity [U]	Product concentration [U ml^{-1}]
1.450	1.750	2.400	24 874	119 400
1.800	2.125	2.685	20 882	112 133
Control[a]	1.600	1.595	890	2800

[a] No resuspension.

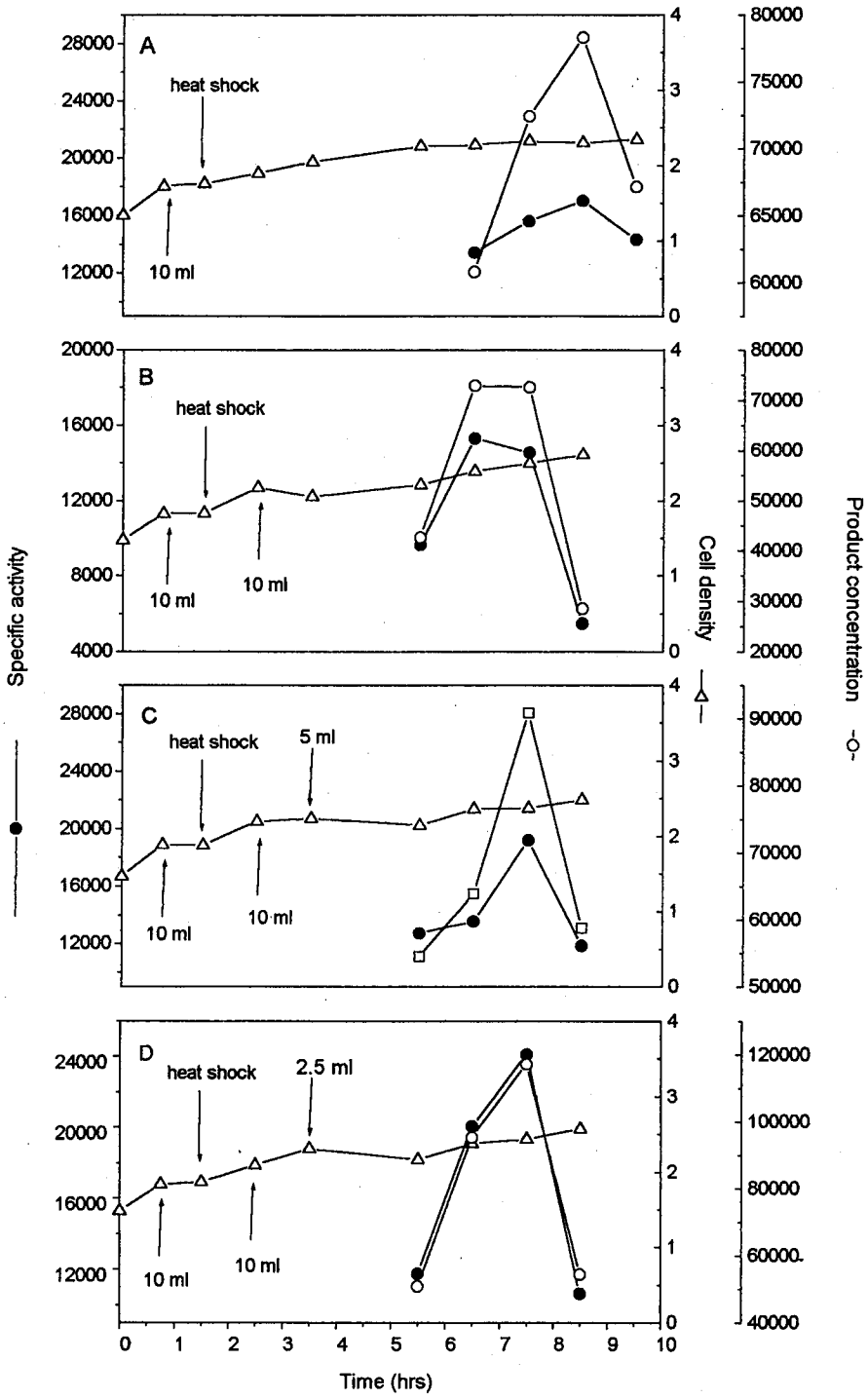

Fig. 5. Time profiles of cell density (g l^{-1}), specific activity (U) and product concentration (U ml^{-1}) in shake flask cultures using fed batch techniques. The arrows indicate the different time points in which either heat shock was given or concentrated 5 × medium was added.

Table 2
Effect of addition of concentrated 5× medium on β-galactosidase expression

Cell density of culture (X_T) [g l^{-1}]	Volume of 5× medium added [ml]	Heat shock given at (X_T) [g l^{-1}]	X_T after 4 h induction [g l^{-1}]	Specific activity [U]	Product concentration [U ml^{-1}]
1.400	2	1.550	2.110	23 760	100 266
1.850	2	2.000	3.050	18 409	112 300
1.500	0 (control[a])	1.600	1.595	890	2800

[a] No addition of fresh medium.

ing two feeds gave a better specific activity of 24 000 U as compared to 15 300 U for a single feed. The maximum product concentration was also ~60% higher as compared to a single feed (Fig. 5B–D). The above results indicated that a continuous supply of nutrients more or less matching the uptake of nutrients by the growing cells provided the optimum conditions for recombinant protein production. It was therefore decided to do continuous feeding in a bioreactor and monitoring the effect of different feeding strategies on recombinant protein expression.

In order to maintain nutrient quality in the bioreactor it was decided to increase the feed rate stepwise in an exponential fashion. This would match the uptake of nutrients by the growing cells, thereby keeping them in an extended exponential growth phase even at high cell density. However, a fast specific growth rate may cause an acetate build up which would be deleterious to both cell growth and recombinant protein expression (Jensen and Carlsen, 1990; Yee and Blanch, 1993b; Sakamoto et al., 1994; Shimizu et al., 1992; Kwon et al., 1996). Therefore the pH of the culture was monitored and the data used to control the feeding rate which was appropriately lowered whenever the pH started falling since it was an indication of acetate build up.

The other bioprocess parameter, which was monitored, was the agitation rate. Since dissolved oxygen tension in the bioreactor was controlled by the agitation rate, the agitation rate (rpm) was an extremely sensitive parameter for estimating the oxygen uptake rate (OUR) of the cells. As a stochiometric relationship exists between OUR and cell growth rate, the agitation rate provided us an on-line estimate of the growth rate. Therefore, the feeding rate was only increased when there was a concomitant increase in the agitation rate.

Initially the feeding strategy was tested without induction. At the start of the experiment 1 l medium was taken in the bioreactor and inoculated by a 50 ml overnight culture. Feed (5× medium) was started when the cell density was ~1.5 g l^{-1} at the rate of 55 ml h^{-1}. However, since the pH started to decline, the feed had to be reduced in steps to 20 ml h^{-1}. After the pH stabilized, the feed was increased stepwise to 140 ml h^{-1} in an exponential fashion. The agitation rate also increased during this period demonstrating the increase in the OUR of the culture. This exponential phase of growth lasted until the cell density reached ~10 g l^{-1} after which there was very little increase in the cell concentration (Fig. 6). The agitation also did not increase beyond this point and therefore the feed was kept constant at 150 ml h^{-1}.

For getting recombinant protein expression, a similar feeding strategy was used. Heat shock was given for 2 min, when the cell density was 5.2 g l^{-1} and the cells were in exponential phase of growth. It was observed that the agitation rate remained around 500 and did not increase further after the heat shock. This was possibly because the specific growth rate declined due to the metabolic burden associated with recombinant protein expression. The feed rate was therefore also not increased further and maintained at 60 ml h^{-1}. A maximum activity of 285 000 U ml^{-1} (equivalent to 0.95 g l^{-1} of soluble β-galactosidase) was observed 8 h after heat shock (Fig. 7).

This corresponded to a cell density of 7.1 g l^{-1} and a specific activity of 20 070 U. The above experiment demonstrated that it was possible to maintain a high specific activity of β-galactosidase corresponding to 25% of total protein even at high cell densities provided the cells were maintained in the exponential phase of growth at the time of induction. The effective gain in final product concentration is summarized in Table 3.

A decline in activity was observed 10 h after heat shock in the fed batch experiment, which is similar to those, observed during shake flask experiments. To check whether this was due to a drop in RNA polymerase level, a second heat shock was given to reinduce the λP_L promoter. However, no significant increase in the β-galactosidase activity was observed (data not shown), which indicated that the fall in activity of β-galactosidase was not due to any drop in the levels of T7 RNA polymerase. However, with media containing glycerol instead of glucose, sustained high level expression of β-galactosidase was observed

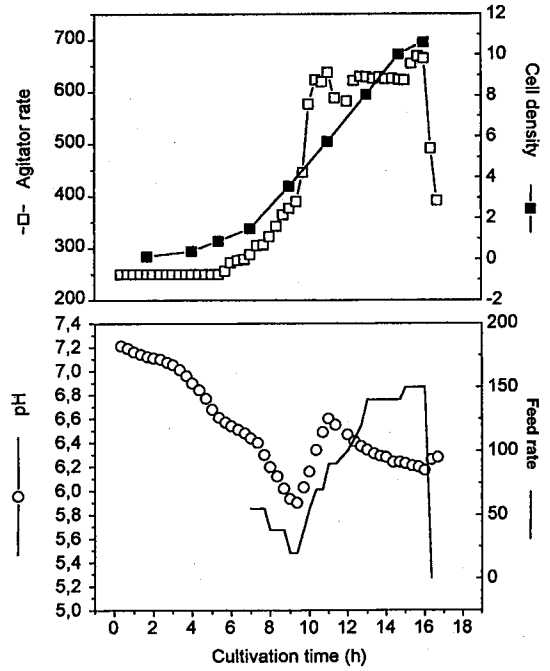

Fig. 6. Time profile of cell density (g l^{-1}), agitation rate (rpm), pH and feed rate (ml h^{-1}) in fed batch culture without induction.

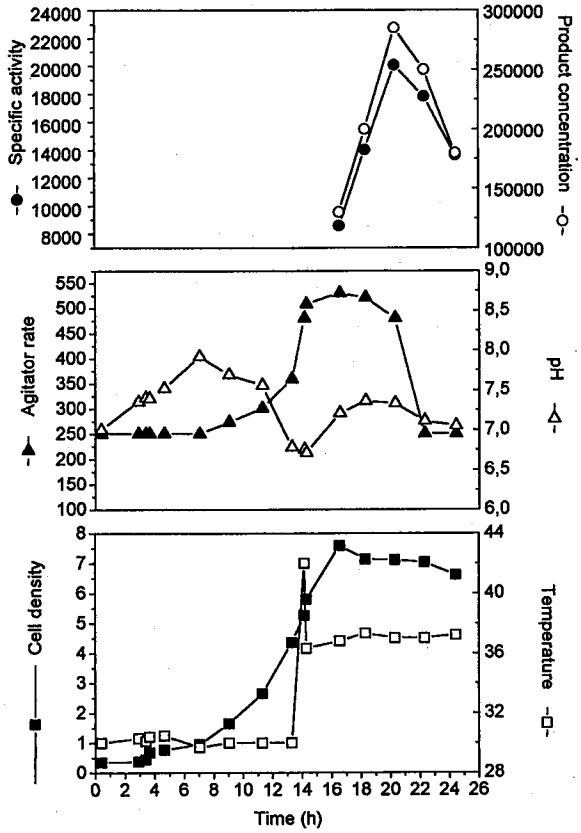

Fig. 7. Time profile of cell density (g l^{-1}), specific activity (U) and product concentration (U ml^{-1}) along with various bioprocess parameters in fed batch culture with induction at $X_T = 5.2$ g l^{-1}.

(manuscript in preparation). Thus media constituents seem to play a vital role in sustaining the high level expression of the recombinant protein.

Attempts to get the same specific activity at even higher cell densities failed primarily since the exponential growth could not be sustained at high cell density. For this, a much more stoichiometrically correct medium needs to be used for the experiments.

4. Conclusion

We were able to demonstrate that the T7 promoter can lead to a high level of recombinant protein expression by proper control of biopro-

Table 3
Cell density (X_T), specific activity and final product concentration achieved with different cultivation strategies

Culture volume	Medium used	Cell density (X_T) [g l^{-1}]	Specific activity [U]	Product concentration [U ml^{-1}]	Reference
10 ml	LB	0.375	22 500	16 875	Fig. 3
75 ml	LB modified	2.430	24 115	117 196	Fig. 5
1.5 l	LB modified	7.100	20 070	285 000	Fig. 7

cess parameters. Since induction was done by temperature upshift and no chemical inducer was used, this strategy has the potential of reducing the fermentation cost and simplifying the downstream processing. It was found that heat shock duration sufficient for suboptional not maximal expression of T7 RNA polymerase is required for high recombinant protein expression. Substrate feed, which maintained nutrient quality and also prevented acetate build up, helped to increase the exponential phase of growth thereby allowing induction at high cell density (5.2 g l^{-1}). This in turn helped in achieving a final product concentration of 0.95 g l^{-1} of soluble recombinant protein.

Appendix A. Nomenclature

X_T total dry cell weight (g l^{-1})

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EXHIBIT D

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MicroReview

The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*

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Summary

At the onset of starvation *Escherichia coli* undergoes a temporally ordered program of starvation gene expression involving 40–80 genes which some four hours later yields cells possessing an enhanced general resistance. Two classes of genes are induced upon carbon starvation: the *cst* genes, requiring cyclic AMP, and the *pex* genes, not requiring this nucleotide for induction. The *cst* genes are not involved in the development of the resistant state and are concerned with escape from starvation, while the *pex* gene induction appears to be associated with resistance. Many of the latter are induced in response to a variety of starvation conditions. They include heat shock and oxidation resistance genes, and some utilize minor, stationary-phase-specific sigma factors for induction during starvation. The protective role of stress proteins may be due to their ability to rescue misfolded macromolecules. The starvation promoters can be potentially useful for selective expression of desired genes in metabolically sluggish populations, e.g. in high-density industrial fermentations and *in situ* bioremediation.

Introduction

Bacteria in nature are often exposed to extreme nutrient scarcity. In aquatic environments individual carbon substrate concentration can be as low as 6–10 $\mu g\ l^{-1}$, much of which may not be biodegradable because of association with aquatic humus (Morita, 1988). Consequently, depending on the environment, slow growth, or intermittent periods of rapid growth interspersed with prolonged periods of non-growth and starvation are the norm for bacteria in nature: the mean generation time in such environments can be as long as 210 days. In host tissues as well, bacterial growth appears to occur at submaximal rates, and starvation and other stresses apparently play important roles in the pathogenic process (Matin *et al.*, 1989). *Escherichia coli* thrives as a commensal/pathogen of a vertebrate host, or in soil and water. It has a doubling time of about 20 hours in the intestine (Gibbons and Kapsimalis, 1967) and survives almost indefinitely in extremely nutrient-poor soils and waters (Carillo and Hazen, 1985); it is therefore a good model system for studying bacterial starvation physiology. Certain bacteria (e.g. *Bacillus* and myxobacteria) form highly resistant dormant structures when subjected to nutritional stress. This strategy trades off one survival mechanism for another: rapid growth under favourable conditions (albeit accompanied by greater sensitivity to stresses) versus dormancy and resistance under stressful conditions, which ensures survival even if multiplication and colonization are precluded. This differentiation requires complex molecular machinery. However, the vast majority of bacteria, including *E. coli*, do not show corresponding morphological development in response to nutritional stress. I review here the studies that indicate that a molecular programme of gene expression is triggered by nutrient deprivation also in *E. coli*, leading to the formation of more resistant cells. There have been similar findings for *Salmonella* (Spector *et al.*, 1988) and marine *Vibrio* (Nystrom and Kjelleberg, 1989); these are reviewed elsewhere (Matin *et al.*, 1989).

A study of stationary-phase bacteria is relevant also for numerous applied purposes. It is in this phase that bacteria form many valuable products such as antibiotics and enzymes of industrial importance. But even for the expression of growth-associated activities of industrial interest, it is desirable that essentially resting cells, preferentially expressing activities of interest, be used. This strategy prevents wasteful diversion of nutrients in biomass formation, decreases nutrient demand and biomass proliferation, and permits the use of compact, high-density bioreactors (Inloes *et al.*, 1985). As discussed below, the starvation promoters may make it feasible to obtain high-level expression of desired structural genes in slow or non-growing bacterial populations.

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A

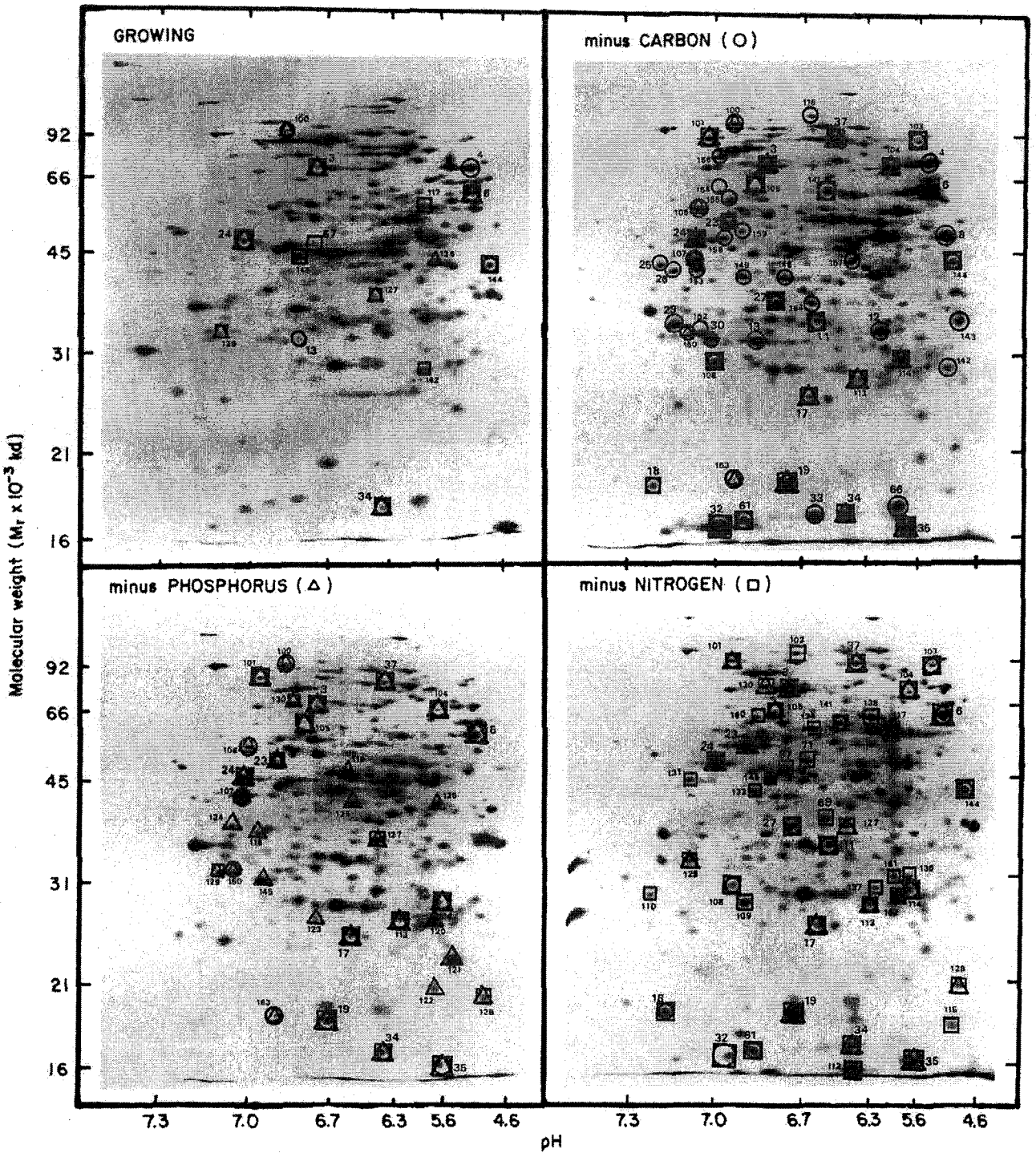
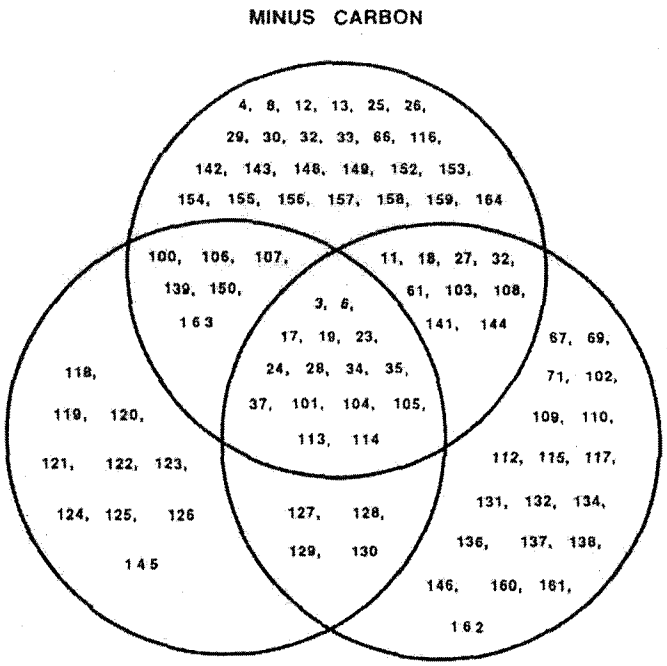


Fig. 1. A. Two-dimensional gel electrophoresis maps of protein synthesis in *E. coli* K12 subcultures during growth and at 30 min of starvation for carbon, phosphate or nitrogen. The subcultures were sampled and pulse-labelled as described in the text (Groat *et al.*, 1986). Easily visualizable polypeptides induced by individual starvations are indicated by the following symbols: ○, carbon starvation; □, nitrogen starvation; △, phosphorus starvation. Numbers below 100 correspond to the numbering scheme we used previously to designate individual polypeptides; those above 100 designate additional polypeptide spots we have detected on these gels following their original publication. Reproduced with modifications from Groat *et al.* (1986).



B. Ven diagram based on (A) to show unique and overlapping polypeptides induced in response to *different individual starvation conditions.*

At the onset of starvation, bacteria degrade cellular proteins rapidly (Mandelstam, 1963); our studies strongly suggested that the amino acids that resulted from this degradation were then utilized to form new proteins unique and beneficial to the starvation state. *E. coli* mutants CM17 and CM89 (Miller, 1975), which were deficient in protein degradation during starvation, also demonstrated impaired bulk protein synthesis and survival when starved (Reeve *et al.*, 1984a). The defect in protein synthesis arose from amino acid scarcity, since exogenous amino acids restored this capacity; even the wild-type bacteria appeared better able to develop starvation resistance if protein synthesis was facilitated by provision of exogenous amino acids to the starving culture (Bockman *et al.*, 1986).

The starvation protein synthesis was, however, not necessary for short-term survival. Wild-type *E. coli* cells starved for glucose continue to synthesize bulk protein at about 80% of the rate of the growing cells for the first three to four hours (see Fig. 2). Essentially complete inhibition of this synthesis (e.g. by chloramphenicol addition) during this period had no immediate effect on culture viability. However, in later phases of starvation the rate of loss of viability was greater the earlier in starvation protein synthesis was inhibited. Thus, although the cells could maintain viability in the virtual absence of protein synthesis, their ability to survive long-term starvation depended on the proteins synthesized in early starvation; some four hours of synthesis of starvation proteins was required for maximal resistance. Use of appropriate mutants showed that the chloramphenicol effect was not mediated through the stringent response, and other approaches for inhibiting protein synthesis gave similar results (Reeve *et al.*, 1984b; Matin *et al.*, 1989).

The starvation proteins

The O'Farrel two-dimensional polyacrylamide gel electrophoresis technique, which reveals the protein synthesis profile of cells, directly demonstrated the starvation proteins (Groat *et al.*, 1986). Cells were grown in a mineral salts medium in which growth stopped because of the exhaustion of a known nutrient. Aliquots of the culture were analysed during growth and at various times during starvation. Starvation for an individual nutrient either initiated or increased the synthesis of several proteins: induction of some 55, 35, and 47 polypeptides can be visualized under carbon-, phosphorus-, or nitrogen starvation, respectively (Fig. 1). These numbers are underestimates since only the more obvious spots are taken into account, the particular procedure used does not detect all classes of cellular proteins, and at a given time of starvation (30 min in this case) not all of the starvation proteins are present simultaneously. It is clear, however, that proteins unique to a starvation condition, as well as common to others, are induced, and some 15 polypeptides constitute a core set that is induced under all the starvation conditions examined (Fig. 1B).

The temporal pattern of synthesis of some 20 of these proteins was analysed. Gels were prepared from samples

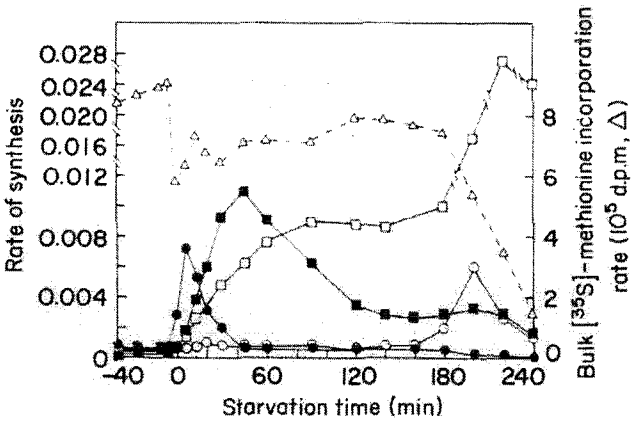


Fig. 2. Rate of synthesis of individual polypeptides in an *E. coli* K12 culture entering stationary phase because of glucose exhaustion. All the kinetic classes observed are included; there were three individual starvation proteins in each of the classes represented by polypeptides numbers 9 (●), 14 (○), and 3 (■), while 11 proteins exhibited kinetics similar to that of polypeptide number 19 (□). The bulk protein synthesis rate is also shown. Note the difference in scale of bulk protein synthesis relative to the scale of individual polypeptides. Reproduced with modification from Groat *et al.* (1986).

removed at different times from a starving culture, and each starvation polypeptide was quantified by radioactivity measurement or computer-assisted densitometry. Of these, 11 exhibited the kinetic pattern illustrated by polypeptide 19 (Fig. 2); of the remaining three, each fell into the remaining classes shown. Essentially the same kinetic pattern was observed in cultures entering the stationary phase because of succinate or nitrogen exhaustion. Most of the core set of proteins exhibited kinetics of synthesis similar to polypeptide 19.

The Cst and Pex proteins and starvation survival

Genetic studies (see below) revealed that some of the carbon-starvation genes required cyclic AMP (cAMP) for their induction while others did not. Two-dimensional gel analysis of carbon-starvation protein synthesis in a Δ*cya* (or Δ*crp*) mutant showed that of the 30 proteins analysed, 19 required cAMP and the rest did not: in the mutants, the former class was not induced at all upon glucose starvation, while some of the latter were hyperinduced. These have been termed Cst and Pex proteins, respectively. It is noteworthy that all of the core set of proteins belong to the Pex class (Fig. 1B). Since a starved Δ*cya* mutant induces only the Pex proteins, it should exhibit increased sensitivity to starvation if Cst-protein induction has a role in starvation survival. However, the deletion and the wild-type strains showed the same resistance to starvation; thus, induction of Cst proteins has no protective role. As discussed below, increased levels of σ^{32}, encoded by the *rpoH* gene, are required for the synthesis of some of the Pex proteins during starvation, e.g. DnaK (spot 4; Fig. 1), GroEL (spot 6), and HtpG (not prominent in 30 min gels shown). Induction of these proteins may be important in starvation survival, since in two separate experiments the Δ*rpoH* strain showed greater sensitivity to starvation (Jenkins and Matin, 1990). These experiments were conducted at 20°C because the deletion strain cannot grow above this temperature. Whether induction of these proteins would enhance resistance at other temperatures is not known. The fact that most Pex proteins belong to the core set of proteins appears consistent with their protective role since it would be economical to use the same mechanism for stress resistance regardless of the identity of the limiting nutrient.

The Pex proteins and cross-protection

Some 11, 6, and 5 starvation proteins are common to proteins induced in *E. coli* by heat shock, oxidative stress or osmotic stress, and of these, eight, five, and five, respectively, are Pex proteins. Carbon- or nitrogen-starved *E. coli* cells survive exposure to a lethal dose of heat (57°C) much better than their exponential-phase counterparts, and the degree of resistance increases with the duration of starvation. After seven minutes of exposure, cells obtained from the growing culture were completely non-viable, whereas cells starved for one, two or four hours exhibited 40, 70, and 85% survival, respectively (Jenkins *et al.*, 1988). Longer starvation did not enhance resistance any further. No resistance developed during starvation in the presence of chloramphenicol. None of the Cst proteins common with heat-shock proteins played a role in this phenomenon, since the Δ*cya* strain developed heat resistance upon starvation in precisely the same manner. Pex-dependent progressive resistance during starvation developed also to oxidation, osmotic (Jenkins *et al.*, 1990) and, as found recently, to acid stresses: cells from a growing culture exposed to pH 5 lost 100% viability within about 20 hours, but a three-hour-starved counterpart of this culture was still >40% viable (M. Keyhan and A. Matin, unpublished). In all cases examined, starvation cross-protected against other stresses to a greater degree than pre-adaptation with the particular stress itself.

Functions of starvation proteins

We speculated that the Cst proteins which, as we have seen, are not concerned with the cellular resistant state, were concerned with enhancing the cell's metabolic potential (Matin *et al.*, 1989). Their dependence on cAMP is consistent with such a role since many genes requiring this nucleotide encode proteins involved in uptake and catabolic functions. The recent cloning of the *cstA* gene

provided a direct test of this hypothesis (Schultz and Matin, in press). It encodes a highly hydrophobic protein, and λ*plac*Mu9 insertions in this gene interfere with *E. coli* growth on peptides as the carbon and energy source. Thus the induction of *cstA* during the dearth of readily catabolizable substrates would increase the likelihood of *E. coli* escaping starvation by using alternate growth substrates.

As stated above, the Pex proteins include many heat-shock proteins whose function has been receiving increasing attention. These proteins are highly conserved through evolution: the prokaryotic DnaK is homologous to the eukaryotic Hsp70 family, GroEL has its counterpart in eukaryotic mitochondrial Hsps as well as the plant ribulose biphosphate carboxylase (RuBisCO)-binding protein, and HtpG is homologous to eukaryotic Hsp83 (Morimoto *et al.*, 1990). Several studies point to a role for the major stress proteins in protein folding (foldase function) and macromolecular assembly ('chaperone' function). In *E. coli*, overproduction of DnaK *in vivo* tended to solubilize an improperly folded, precipitated protein (P. Blum, M. Velligan, N. Lin, and A. Matin, submitted). In both prokaryotes and eukaryotes the unfoldase action is necessary for membrane transit-competence of proteins. The stress proteins bind to nascent polypeptides destined for translocation before the latter fold; alternatively, they may bring about an ATP-dependent unfolding of such proteins. ProOmpA, diluted from 8 M urea, remained translocation competent with or without GroEL but maintained this competence for more than two hours only in the presence of the chaperone (Morimoto *et al.*, 1990). Goloubinoff *et al.* (1989) reconstituted active prokaryotic RuBisCO, a dimeric protein, from unfolded polypeptides, which bound to GroEL. ATP and GroES were required to bring about the release of the enzyme subunit in folded form. Disassembly of reaction complexes also involves stress proteins. DnaK, DnaJ and GrpE proteins disassemble the tight *ori*λDNA–λO–λP–DnaB complex, liberating DnaB helicase required in λDNA replication; the DnaK eukaryotic homologues, the Hsp70 proteins, disassemble clathrin triskelions from coated vesicles; like DnaK, the Hsp70 may require the eukaryotic homologues of DnaJ and GrpE for optimal function (Morimoto *et al.*, 1990). It is possible that the stress proteins can non-specifically bind to any unstructured or improperly folded polypeptide to unfold or promote folding as may be required for specific functions and conditions and for facilitating reactions involving multiple proteins. It is therefore not surprising that null mutations in genes encoding the stress proteins are either lethal or have a highly pleiotropic phenotype, causing defects in cell division, RNA and DNA replication, and proteolysis (Morimoto *et al.*, 1990); Raina and Georgopoulas, 1990). Stress would tend to promote abnormal conformation of macromolecules and increased levels of stress proteins would therefore have a protective effect. Recent studies indicate that stress proteins tend to be the major target of the immune response in certain infections (Young *et al.*, 1990).

cst and *pex* gene regulation

We have isolated some twenty *lacZ* fusion strains in the *cst* genes (and three in the *pex* genes) using Mu dX or λ*plac*Mu9 phages. As illustrated for *cstA* and *pexA*, the *lacZ* gene in these fusions is under the control of the respective starvation-response regulatory region (Fig. 3). The *cstA* gene is not expressed in a Δ*cya* background either during growth or starvation; in contrast, the *pexA* gene exhibits increased levels of expression in this background both during growth and starvation (Fig. 3). We have cloned the regulatory region of several of these genes by reconstitution of a truncated plasmid *lacZ* gene by supplying the missing *lacZ* fragment, along with the *cst* or *pex* regulatory region, from the genomic DNA of the fusion strain. The *cstA* gene can be fully induced in exponential-phase cultures by the addition of exogenous cAMP. Cell-free S30 coupled transcription-translation studies revealed the requirement for σ^{70} RNA polyermase and cAMP but no additional stationary-phase-specific factor (Blum *et al.*, 1990). By map location restriction site and sequence comparisons, we located the *cstA* regulatory sequence in the 180 bp region immediately downstream of *entCEBA*-P15 operon previously cloned by Nahlick *et al.* (1987). The *ent* genes encode the biosynthetic enzymes for enterochelin and are induced by iron deficiency, but

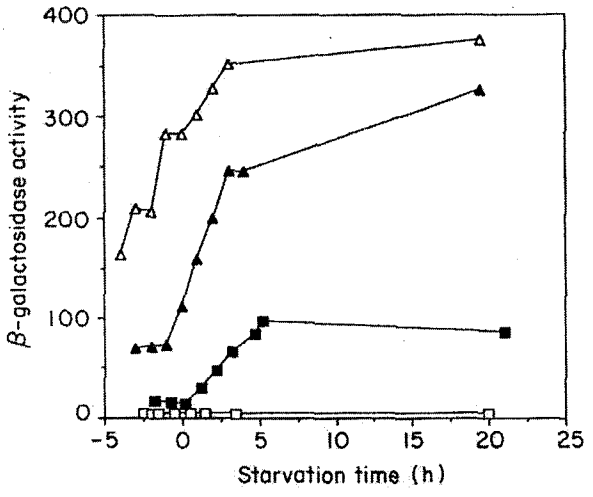


Fig. 3. β-galactosidase activity of *E. coli cstA::lacZ* (■, □) and *pex::lacZ* (▲, △) fusion during growth and starvation in a *cya*$^+$ (solid symbols) and a *cya*$^-$ (open symbols) background. Zero time denotes the onset of glucose starvation. The *cstA* gene maps at 14 and *pex3* at 42 min. A similar induction pattern occurs during starvation for a variety of other carbon substrates. *cstA* data are reproduced from Schultz *et al.* (1988); *pexA* data are from the unpublished work of E. Auger and A. Matin. The scale on the ordinate should be multiplied by 10 for (■).

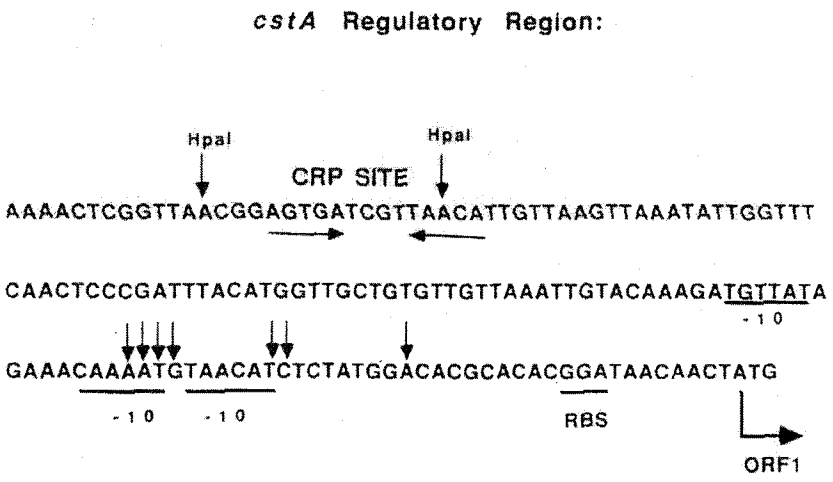


Fig. 4. The *cstA* regulatory region. Reproduced from Schultz and Matin (in press).

cstA is not iron-starvation-inducible. Primer extension analysis showed three sites of varying strengths for carbon-starvation-inducible transcription (Fig. 4); these transcripts are not observed in growing cells. In agreement with the S30 results, upstream of each of the three start sites is an $E\sigma^{70}$ −10 sequence. A putative CRP-binding site is 80 nucleotides upstream of the strongest transcriptional start site; its partial removal abolishes transcription from all three start sites. $E\sigma^{70}$ promoters are typically activated over short distances and this may be the reason for the greater strength of the start site nearest to the CRP-binding sequence. Three open reading frames were found downstream of this *cst* regulatory region encoding proteins of 562, 138, and 65 amino acids, respectively. Carbon-starvation-inducible expression of proteins of this size from the cloned DNA was demonstrated using maxicells. All three proteins may be involved in peptide transport, as discussed above (Schultz and Matin, in press).

Unlike the *cstA* regulatory region for whose full activation the increased cAMP levels of the stationary phase may be sufficient, other *cst* gene induction probably requires additional factors. This is suggested by the *cst8* gene induction pattern in a *crp**, Δ*cya* mutation background. *crp** makes Crp partially independent of cAMP, and the absence of cAMP in this strain ensures the uniform stimulation of cAMP-dependent promoters during growth and starvation. Thus, in such a background any stimulation of a *cst* promoter upon starvation (as was observed for *cst8*) is indicative of additional stationary-phase-specific factor(s) (Blum *et al.*, 1990). Similarly, the *cstC* gene did not switch on in coupled transcription-translation assays conducted with S30 extracts of exponential-phase cells even in the presence of high levels of cAMP. Work on the sequence of the regulatory region of these and the cloned *pex* genes is in progress to determine whether unique promoter/enhancer sequences are present.

Role of stationary-phase-specific σ factors

Given the global nature of change in gene expression in the stationary phase, the involvement of minor σ factors can be expected, and indeed evidence for this has recently been mounting. As noted above, the Δ*rpoH* strains failed to induce some of the heat-shock Pex proteins during carbon starvation. Western blot analysis showed that the concentration of σ^{32} increased during starvation (D. Jenkins, and A. Matin, submitted). It is not known whether this increase is the result of transcriptional, translational, or another type of regulation, e.g. stabilization of message or protein, etc. The *rpoH* regulatory region is complex and contains at least four promoters which appear to be expressed under different conditions. Three of these promoters are $E\sigma^{70}$-driven, and one is driven by $E\sigma^{24}$. One of the former contains a Crp site and may be responsible for the increased transcription of σ^{32} during carbon starvation. However, Nagai *et al.* (1990) were unable to find increased transcription from this promoter during carbon starvation. Involvement of σ^{32} in heat-shock protein induction during starvation is consistent with the general pattern since other stimuli inducing these proteins (heat shock, ethanol, λ infection, inactivation of 4.5S RNA) also act via increased σ^{32} levels (Gross *et al.*, 1990; Bourgaize *et al.*, 1990).

Another putative σ factor, encoded by the *katF* gene, might be involved in starvation-specific induction of genes concerned with oxidative protection. As discussed above, starved cells are more resistant to oxidative stress, and recent studies suggest that the basis for this resistance is different from that of H_2O_2-induced resistance. The latter

protection is mediated via *oxyR*, whereas stationary-phase-elicited protection involves the *katF* gene instead, which mediates expression of the *xthA* gene (encoding exonuclease III), and the *katE* gene (encoding hydroperoxidase II). The *katF* gene product is homologous to other σ factors (Mulvey and Loewen, 1989).

Connell *et al.* (1987) reported a unique −10 region (CGGCNAGTA) in the microcin-encoding gene of *E. coli*, which is induced in the stationary phase. More recently, Aldea *et al.* (1989) found a similar −10 sequence in the *E. coli bolA* gene (which encodes a morphogen responsible for spherical morphology) and the *ftsQZ* operon (involved in cell division); these genes were induced in stationary phase. A GenBank search picked up several homologues of this sequence, suggesting that genes such as those encoding ribosomal proteins, the α, β, and β' subunits of RNA polymerase, and σ^{70} were stationary-phase genes. However, not all the genes containing this sequence are necessarily expressed in the stationary phase, e.g. the *folA* gene (encoding dihydrofolate reductase). These studies were carried out in complex media and it is not clear what nutrient exhaustion caused growth stoppage. Nonetheless, it is possible that the unique −10 region sequence is recognized by an additional minor σ factor species of RNA polymerase that is found primarily in the stationary phase.

Biotechnological applications of *cst* promoters

I have discussed above the desirability of using low metabolic activity-dense microbial configurations in industrial fermentations. However, the use of growth-dependent promoters to drive product formation presents several problems in such configurations. For example, plasmid instability arises because of negative selective pressure during growth, and it becomes necessary to couple the growth and production phases, causing nutrient diversion away from product formation. The *cst* type of promoter permits separation of growth and production phases, avoids plasmid instability, and permits selective expression of genes of interest in a metabolically sluggish population. We have used the *cstA* regulatory region (Fig. 4) to express heterologous proteins in *E. coli*. Plasmid AMC28 contains the *cstA* promoter transcriptionally fused to the human growth hormone gene; the *E. coli* strain containing this plasmid showed a four-fold induction of human growth hormone in the stationary phase (P. Blum, N. Linn and A. Matin, unpublished).

Preferential expression of desired genes in metabolically sluggish bacteria can also be useful for *in-situ* bioremediation. As discussed above, bacteria in nature typically exist in a state of low metabolic activity. Thus, the bioremediation capacities of natural microflora are expressed at such a low rate that they are often of little practical value, and nutrients have been injected into ground water to enhance degradation of toxic compounds (Vogel *et al.*, 1987). However, apart from the technical difficulties, the rapid microbial growth in the aquifers can have unforeseen consequences and starvation promoters may prove useful in overcoming these problems. We have constructed a plasmid in which the expression of toluene monooxygenase (TMO) gene of *Pseudomonus mendocina* is driven by the *cstC* promoter. TMO can degrade trichloroethylene (TCE) and the *E. coli* strain containing this plasmid degraded TCE primarily in the stationary phase (D. Little and A. Matin, unpublished).

Conclusion

Starvation is a serious problem for bacteria. Not only is it encountered very often in nature, but starved bacteria are also likely to become simultaneously less competent to deal with other stresses. The diminished redox potential, energy status, biosynthetic machinery, and the lowered cytoplasmic pH of starved cells must impair their capacity to mount an effective stress and repair response. This may be why starved bacteria develop a resistant state that is broader and stronger than that elicited by exposure to sublethal doses of other stresses. It appears probable that heat-shock proteins play a role in the development of this resistance, but what these and additional proteins are, how they enhance cellular resistance, and how their induction is triggered by starvation, are questions to which answers are only just beginning to emerge.

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EXHIBIT E

Improvement in Recombinant Protein Production in ppGpp-Deficient *Escherichia Coli*

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Absstract: Maintaining a metabolically productive state for recombinant *Escherichia coli* remains a central problem for a wide variety of growth-dependent biosynthesis. This problem becomes particularly acute under conditions of minimal cell growth such as fed-batch fermentations. In this, we investigated the possibility of manipulating the protein synthesis machinery of *E. coli* whereby synthesis of foreign proteins might be decoupled from cell growth. In particular, the effects of eliminating intracellular ppGpp on the synthesis of foreign proteins were studied in both batch and fed-batch operations. A significant increase in CAT production was observed from the ppGpp-deficient strain during both exponential and fed-batch phases. The increase in CAT production during exponential growth was accompanied by a simultaneous increase in CAT mRNA levels. Interestingly, CAT production was increased five-fold, while the level of CAT-specific mRNA increased only three-fold. Thus, eliminating intracellular ppGpp appears to have increase the production of recombinant protein by increasing not only the pool sizes of CAT mRNA but also possible alternations in the post-transcriptional processes. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* **53**: 379–386, 1997.
Keywords: ppGpp; recombinant protein synthesis; translational machinery; *Escherichia coli*

INTRODUCTION

Typically, production of recombinant proteins from *E. coli* requires a strong coupling between cell growth and production (Luli and Strohl, 1990; San et al., 1994). Production is severely curtailed under slow-growing conditions due to the intense competition between normal cell function maintenance and recombinant protein production for the limited available metabolic machinery. From a processing respective, this problem becomes particularly acute under conditions of minimal cell growth such as fed-batch fermentations (San et al., 1994).

Recent investigations have revealed that the cellular content of many direct participants in protein synthesis (e.g., initiation factors, elongation factors, even RNA polymerase) are decreased when *E. coli* enters stationary phase as the result of nutrient and energy starvation (Matin, 1991; Matin et al., 1989). In particular, the amount of active ribosome is drastically different between fast- and slow-growing cultures (Dennis and Bremer, 1973). Ribosomes are large ribonucleoproteins that are responsible for the translation of mRNAs, and each ribosome is composed of 52 distinct ribosomal proteins (r-protein) and three kinds of rRNA (Jinks-Robertson and Nomura, 1987). Expression of r-protein can vary by more than 500 times depending on the growth rate (Bremer and Dennis, 1987), and is precisely coordinated with the rate of rRNA synthesis (Gourse et al., 1985; Norris and Koch, 1972). Thus, the rate of rRNA synthesis determines the overall availability of ribosome.

Coordination of rRNA synthesis is a central and complex metabolic task. Under extremely fast-growing conditions, rRNA can constitute over half of the total cellular RNA (Jinks-Robertson and Nomura, 1987). Stringent response has been reported to be responsible for regulation of rRNA synthesis (Cashel and Rudd, 1987). The nucleotide ppGpp is the regulatory signal that appears to repress rRNA synthesis under starvation condition. When *E. coli* is subjected to carbon and amino acid starvation, an immediate consequence is the accumulation of unusual nucleotides, ppGpp and pppGpp. Synthesis of ppGpp is governed by at least two ways. The enzyme ppGpp synthetase I (PSI) encoded by the *relA* gene is responsible for ppGpp synthesis during the stringent response to amino acid deprivation (Block and Haseltine, 1974; Cashel and Gallant, 1969). When growth is slowed by the depletion of a primary carbon source, the stringent response is activated by a pathway that is independent of the *relA* gene (Hernandez and Bremer, 1991). A second enzyme, ppGpp synthetase II (PSII), encode by the *spoT* gene, is responsible to catalyze ppGpp synthesis (Gentry and Cashel, 1996; Hernandez and Bremer, 1991; Xiao et al., 1991).

The synthesis of rRNA is known to be inhibited by ppGpp (Ryals et al., 1982). *In vitro* experiments demonstrated that ppGpp has a direct inhibitory effect on *rrn* transcription (Glaser et al., 1983). Even though the exact mechanism of this control is not yet clear, numerous data suggest that ppGpp affects RNA polymerase selectivity, rendering it unable to initiate transcription at stable RNA promoters (Ham-

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ming et al., 1980; Travers et al., 1982). In addition, ppGpp itself is a potent inhibitor of protein synthesis by reducing the RNA chain growth rate, making mRNA limiting for translation during starvation (Svitil et al., 1993). The inability of starved cells to further synthesize recombinant proteins may also be due to this mechanism.

Cells with null alleles in both the *relA* and *spoT* genes were found no longer to accumulate ppGpp upon amino acid and carbon starvation (Hernandez and Bremer, 1993; Tedin and Bremer, 1992; Xiao et al., 1991). As a result, ribosomes were 10–20% overproduced in the ppGpp-less strains at lower growth rates (Herman and Wegrzyn, 1995; Sorensen et al., 1994; Tedin and Bremer, 1992). Overall, the synthesis rate of translational factors were found to be increased by 5–20 fold, while their expression was almost totally shut-off in wild type cells (Nystrom, 1994).

In this paper, our main goal is to study how production of recombinant protein is affected if the potential ppGpp inhibition is removed by eliminating intracellular ppGpp. Although previous results on ppGpp have clearly demonstrated the importance of this factor in determining the translational potential of *E. coli* during restricted growth, one key question which is critical for the strategy proposed here remains. Due to the complexities of the regulation of protein synthesis, it is possible that ppGpp would have little effect on recombinant protein synthesis. We have conducted experiments outlined below, which addressed this concern. These experiments also entail the first reported effects of ppGpp on recombinant protein synthesis in *E. coli.*

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strains CF1648 (MG1655 (λ^-,F^-)) and CF1693 (same as CF1648 except it is also *spoT* and *relA*) were used in this study (Hernandez and Bremer, 1993). Routine transformations were performed with *E. coli* DH1. Plasmid pKC6 (Chen et al., 1993) which expresses chloramphenicol acetyltransferase (CAT) under control of a *tac* promoter, was used as the CAT expression vector.

Media and Growth Conditions

LB medium (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 3 g/L K_2HPO_4 and 1 g/L KH_2PO_4, pH 7.0) and M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 2.5 g/L NaCl, 3 mg/L $CaCl_2$, 0.2 mL of 1M $MgSO_4.7H_2O$ and 1 mL of 20% Casamino acids) were used for both batch and fed-batch cultivations. For all experiments, media were supplemented with 0.2% glucose as the carbon source. Amplicillin was added at 50 mg/L for selection. For the induction of CAT production, 1 mM IPTG was added unless otherwise described. Shake flask experiments were carried out at 275 rpm in a New Brunswick INNOVA 4000 incubator shaker at 37°C.

Fed-batch fermentations were carried out in a 5 L Bioflo 3000 (New Brunswick Scientific, Edison, NJ) bioreactor. The starting working volume was 4 L. The conditions of operation were temperature, 37°C; agitation, 400 RPM; pH, 7.0; initial air flow-rate, 1.5 L/min. pH was controlled by addition of NaOH and HCl. Foam was controlled by the addition of a 2% anti-foam A solution. The inoculum (1:50) was grown in 100 mL of the same medium in a 250 mL flask for approximately 16 h. The amount of inoculum added to the bioreactor was adjusted to give a starting OD of 0.1. The fed-batch feeding was initiated when the dissolved oxygen (DO) concentration rose from 15–20% to nearly 100%. This indicated that the cells were about to enter stationary phase. A solution of 20% glucose and 10% casamino acids was fed to the bioreactor at a feed-rate of 4 mL/h. After 26 hours post-inoculation, the feed-rate was changed to 8 mL/h and the air flow-rate to 3 L/min. Ampicillin was added continuously to maintain selection pressure throughout the feed-batch fermentation.

Chemicals, Reagents, and DNA Manipulations

All restriction endonucleases, modifying enzymes (T4 DNA polymerase, Klenow fragment, T4 DNA Ligase) and IPTG were purchased from New England BioLabs, Boehringer Mannheim Biochemicals, or Promega. ^{32}P-dCTP was purchased from New England Nuclear. All DNA manipulations were done according to standard methods (Sambrook et al., 1989). DNA fragments were eluted from agarose gels using a Sephaglas BandPrep Kit from Pharmacia. Rifampicin was purchased from Sigma Chemical.

Protein and CAT Assays

Cells were harvested by centrifugation and disrupted by sonication. The soluble fraction was used for protein and CAT analysis. Total protein concentration was determined using a Bio-Rad Protein Assay kit. Total CAT content (g CAT) was measured by a CAT ELISA kit obtained from Boehringer Mannheim. The specific CAT content was expressed as g CAT/ g total protein. CAT activity was assayed in parallel following the procedure described in Rodriguez and Tait (1983). Dilution of samples in TDTT buffer was required for samples with high activity. Kinetic measurements were performed with a Beckman spectrophotometer in a 37°C temperature-controlled sample chamber.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). For SDS-PAGE analysis, cell lysate was boiled for 5 min in gel loading buffer (10% glycerol, 5% 2-mercaptoethanol, 3.3% SDS and 0.5 M Tris, pH 6.8) and then electrophoresed on a 12.5% polyacrylamide gel.

RNA Isolation and Analysis

One mL sample from CF1693 (±1 mM IPTG induction) and CF1648 (induced with 1 mM IPTG) cell cultures were col-

lected at various time intervals (pre- and post-induction). RNA was isolated from each sample (Sambrook et al., 1989), then quantified at 260 nm. Six μg aliquots of total RNA from each sample were glyoxylated (Maniatis et al., 1982), then electrophoresed through 1.3% agarose gels. RNA molecular weight standards (0.24–9.5 Kb RNA Ladder, Gibco-BRL) were included. RNA was transferred to nitrocellulose filters for hybridization analyses (Maniatis et al., 1982).

^{32}P-riboprobes were utilized for detection of chloramphenicol acetyltransferase (CAT) mRNAs. A CAT-containing DNA fragment (Chloramphenicol Acetyltransferase GenBlock®, Pharmacia) was ligated into the *Hind*III site of (pT7T3 19U, Pharmacia) to yield pT3CAT. ^{32}P-labelled riboprobe was prepared from *Pst*I-linearized pT3CAT as described by Rao et al. (1994). Hybridization was performed overnight at 65°C (Maniatis et al., 1982). The stringency used during the final washings of the blot was 68°C; 0.1× SSC. Autoradiographs were scanned using a LKB Ultrascan scanning laser densitometer for quantification of the CAT mRNA. Counts thus obtained were normalized with total RNA in order to obtain a measure of specific gene activity as described before (Chen et al., 1995).

RESULTS AND DISCUSSION

Effect of ppGpp on Recombinant Protein Synthesis

The first set of experiments was designed to explore whether ppGpp plays an important role in recombinant protein synthesis. Comparison of CAT production profiles between ppGpp-less and wild-type strains would provide us this information. *E. coli* strain CF1693, which is totally devoid of ppGpp, and a wild-type control strain CF1648 (MG1655) were employed in these initial studies. Plasmid pKC6, which expresses chloramphenicol acetyltransferase (CAT) under control of a *tac* promoter, was introduced into these strains. The resulting strains were grown in 250 mL shake flasks containing 50 mL of LB medium with 0.2% glucose, and the levels of CAT production were monitored throughout the batch cultivation. These results are depicted in Figure 1. Before induction, CAT production was very low in both strains due to tight regulation of the *tac* promoter. However, upon IPTG addition, the CAT level increased gradually for strain CF1648, while a rapid increase in CAT production was observed for strain CF1693. The amount of CAT eventually leveled off after four hours of induction, and remained relatively constant thereafter. The final CAT level was about 5-fold higher in strain CF1693. These findings are important for several reasons. It demonstrates that ppGpp has a profound effect on recombinant protein synthesis. Secondly, it also illustrates that eliminating ppGpp has a beneficial effect on recombinant protein production even during exponential growth. This observation is unexpected since most reports on ppGpp have indicated differences in protein synthesis only during slow-growing conditions (Nystrom, 1994; Sorensen et al., 1994; Svitil et al., 1993).

Because *relA*$^-$ mutants have unusually high level of amino acid mis-incorporation (Sorensen et al., 1994), in order to ensure that the CAT protein levels measured from the ELISA kit were not the artifacts of inactive or truncated CAT fragments, CAT activity measurements were also performed for selected samples. The resulting CAT activities were in good agreement with the ELISA results, indicating that most of the proteins were indeed active (data not shown). Moreover, selected samples were analyzed by SDS-PAGE as shown in Figure 2. It can be seen clearly that

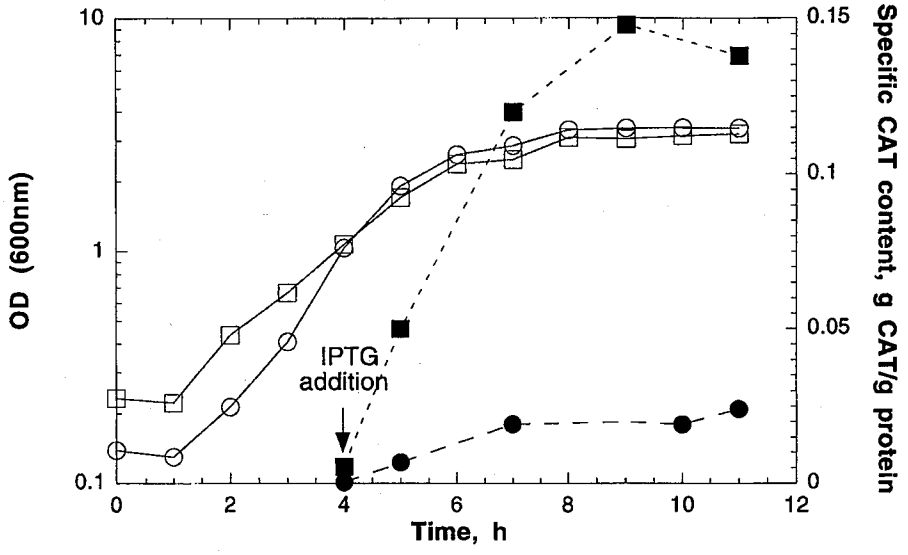

Figure 1. Cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6. Cells were grown in LB medium with 0.2% glucose.

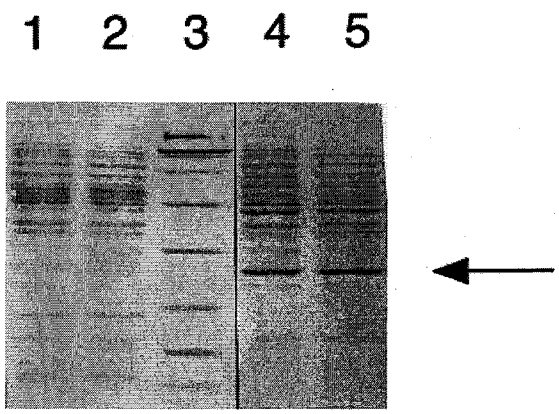

Figure 2. SDS-PAGE of total proteins from strains CF1648:pKC6 (Lane 1 and 2) and CF1693:pKC6 (Lane 4 and 5). Samples were taken at 2 and 4 h post-induction. The molecular weight markers in Lane 3 are as follows: 6.5 kDa, 14.4 kDa, 21.5 kDa, 31 kDa, 45 kDa, 66.3 kDa, 97 kDa, 116 kDa, and 200 kDa. CAT protein bands are indicated by the arrow.

the intensity of the protein bands corresponding to CAT was higher in CF1693, and no other major degraded CAT products were observed. At least under our experimental conditions, amino acid mis-incorporation does not appear to be affecting the production of active CAT products.

A similar enhancement in CAT production was also observed when cells were grown in slower-growing M9 medium. As depicted in Figure 3, there were essentially no differences in cell growth between the two strains. However, the specific CAT level was about 2 times higher in the ppGpp-deficient strain. In contrast to results from LB medium, the major differences in CAT production were observed only when cells shifted into the slow-growing regime with essentially no difference in CAT production during exponential growth. These results suggest that ppGpp affects CAT production differently under different growth conditions, and possibly by different mechanisms.

Effect of ppGpp on Recombinant Protein Synthesis During Fed-Batch Fermentations

Wild-type cells accumulate ppGpp particularly under slow-growing conditions and hence the greatest difference in the amount of ppGpp in cultures of CF1648 and CF1693 would be during slow-growing conditions. Fed-batch fermentations provide a means to create an extended period of slow culture growth to compare the effect of ppGpp deficiency on the production of CAT during extended slow growth.

Cultures of CF1648 and CF1693 carrying pKC6 were grown in a bioreactor as described in Materials and Methods. 0.5 mM of IPTG was added when the culture reached an OD_{600} of about 1.4. Cultures of CF1648/pKC6 were induced 2.5 hours post-inoculation and cultures of CF1693/pKC6 were induced 4.17 hours post-inoculation.

From Figure 4, we see that addition of IPTG induced the production of CAT in both cultures. At the end of exponential phase (~5 hours for CF1648 and ~9 hours for CF1693), cultures of CF1693 accumulated about 4.5-fold higher CAT per total cellular protein compared to CF1648. Thus, expected ppGpp deficiency in late exponential phase results in higher recombinant protein production similar to that observed in batch cultures grown in shake flasks.

Drastic differences in amount of CAT between the two cultures was observed after the initiation of slow feeding of nutrients. At the end of exponential phase, a solution of 20% glucose and 10% casamino-acids was fed to the cultures at a rate of 4 mL/h, when the dissolved oxygen (DO) concentration rose to nearly 100%. The amount of CAT per total protein decreased continuously in cultures of CF1648, even though the total cellular protein increased after feeding was initiated. On the other hand, cultures of CF1693 continued to accumulate CAT after feeding. For example, the amount of CAT per total protein was nearly 70-fold higher in cultures of CF1693 17 hours post-inoculation. Overall, about

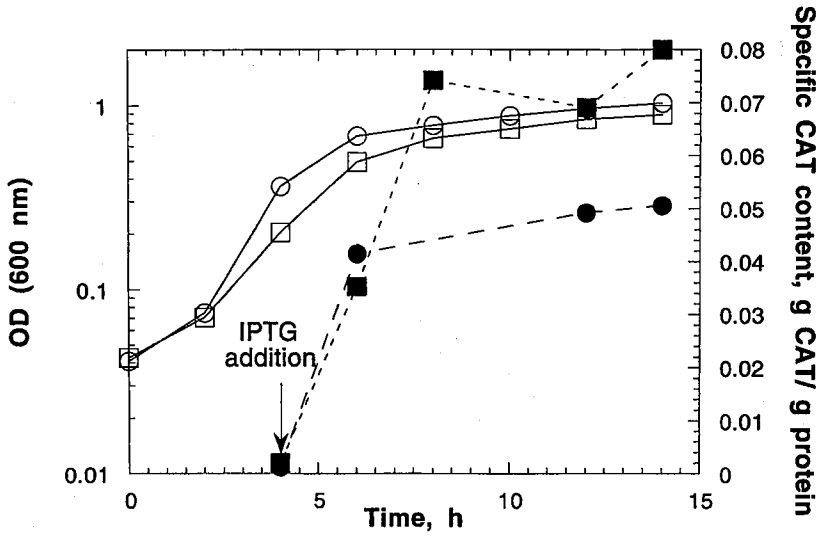

Figure 3. Cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6. Cells were grown in M9 medium with 0.2% glucose.

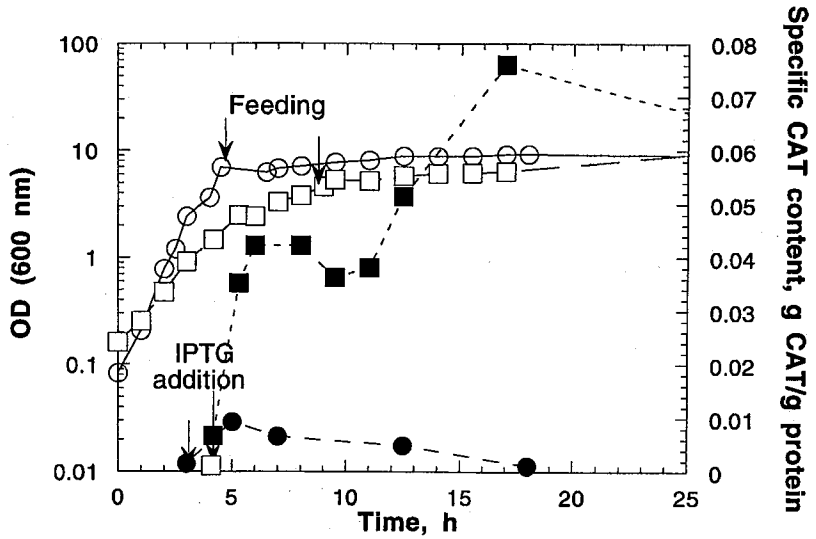

Figure 4. Comparison of cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6 in fed-batch fermentation. Fed-batch fermentations were carried out in a 5 L Bioflo 3000 bioreactor. The starting working volume was 4 L. The conditions of operation were temperature, 37°C; agitation, 400 RPM; pH, 7.0; initial air flow-rate, 1.5 L/min. The fed-batch feeding was initiated when the dissolved oxygen (DO) concentration rose from 15–20% to nearly 100%. A solution of 20% glucose and 10% casamino acids was fed to the bioreactor at a feed-rate of 4 mL/h. After 26 hours post-inoculation, the feed-rate was changed to 8 mL/h and the air flow-rate to 3 L/min. Amplicillin was added continuously to maintain selection pressure throughout the feed-batch fermentation.

20 times as much CAT was produced in the ppGpp-deficient strain during the fed-batch phase.

Comparison of CAT mRNA Level

In wild-type cells, a low level of ppGpp is normally detected during exponential growth (Hernandez and Bremer, 1993), and very little difference in cellular mechanism is expected between strains CF1693 and CF1648. It is therefore uncertain what mechanism is responsible for the increase in CAT production during exponential grown in LB medium. However, in addition to an improved translational apparatus, the rate of mRNA synthesis has also been shown to be drastically higher in ppGpp-less strains, particularly during rapid cell growth (Hernandez and Bremer, 1993; Kingston and Chamberlin, 1981). This could be a possible explanation for our observed results. To investigate this possibility, we have conducted experiments to compare the level of CAT mRNA upon induction. Samples were taken as early as 4 min. post-induction up to 6 h post-induction. As depicted in Figure 5, the level of CAT mRNA was at least 2-fold higher in the ppGpp-deficient strain CF1693, with the most significant difference during the initial 2 hours post-induction. This difference in CAT mRNA levels gradually decreased with time, and the levels were essentially the same between the two strains when cells shifted into stationary growth (~6 h post-induction).

For ppGpp-deficient cells growing exponentially in LB medium, the improvement in CAT production appears to be partially due to an increase in transcript levels. Since little difference was observed between the two strains in CAT-specific mRNA levels during stationary growth (see Figure 5), the observed increase in CAT production in stationary phase cultures of CF1693 does not appear to be attributable to a difference in transcript levels, suggesting that other post-transcriptional mechanisms, such as proteolysis (Harcum and Bentley, 1993) or translation (Hernandez and Bremer, 1993; Nystrom, 1994; Sorensen et al., 1994), may be responsible for the enhancement.

In order to investigate whether differential proteolytic activities might be at least partially responsible for the observed increase in CAT expression in CF1693 versus CF1648, CAT activities were monitored in late log-phase cells in the presence of rifampicin. Cultures of CF1648:pKC6 and CF1693:pKC6 were induced with 1 mM IPTG and incubated until OD_{600} approximately 1.0. 4 mg of rifampicin was then added every 30 minutes to the cultures to terminate translation of new polypeptide chains. Samples were collected every 30 minutes for 2.5 hours and their CAT activities were quantified. No significant decline in CAT activity could be detected in the wild-type strain, while a small decline in activity was observed in strain CF1693 (Fig. 6). These results indicated that proteolysis does not appear to play an important role for the increase in CAT production in the ppGpp-less strain.

The possible enhancement during the slow-growing regime is better indicated in the fed-batch experiment in which the ppGpp-less strain accumulated a much higher level of CAT during the slow-growing fed-batch phase, where there was no significant difference in CAT nRNA levels (data not shown). The specific CAT accumulation rate was about 20-fold higher in the ppGpp-less strain. Along the same line, no difference in CAT production was observed in M9 medium until the stationary phase, because

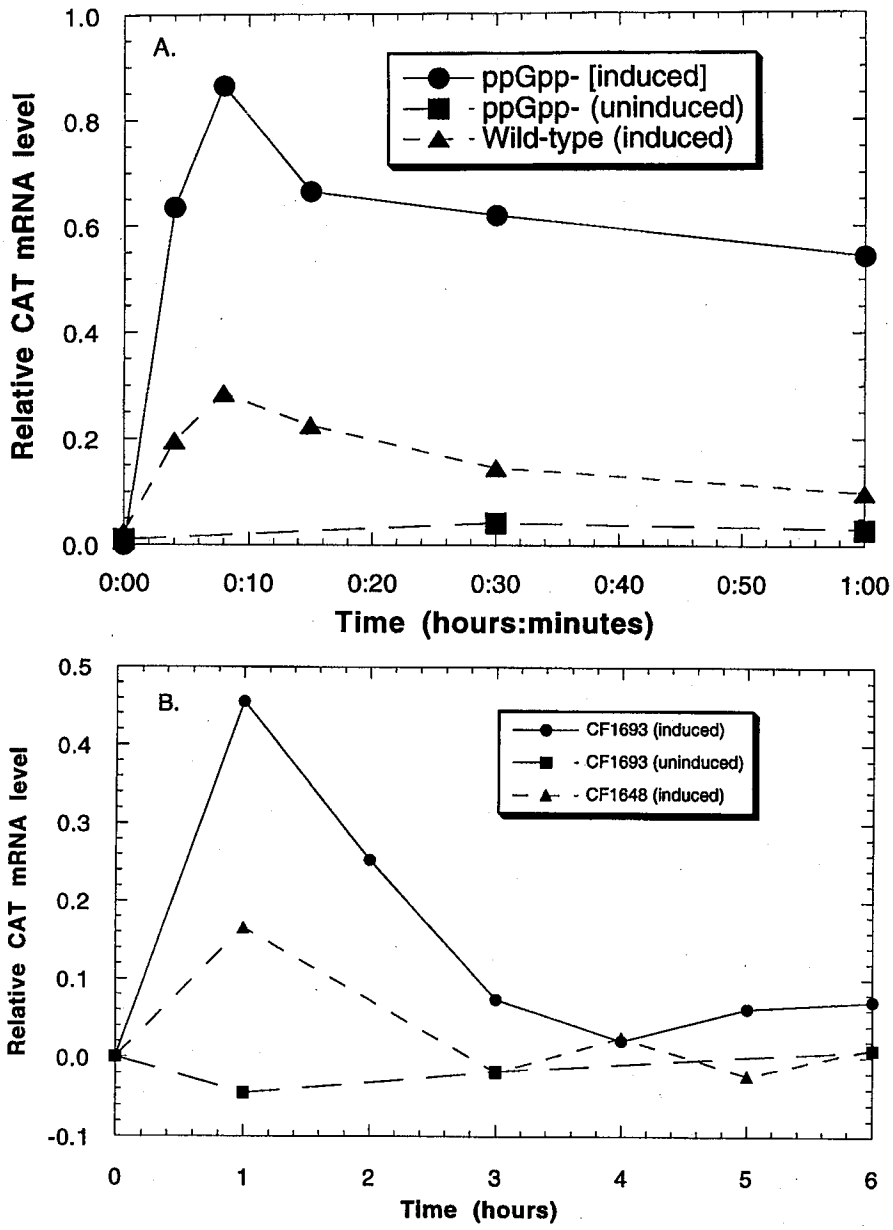

Figure 5. Comparison of CAT mRNA levels between strains CF1648 and CF1693. (A) The initial response during the first hr post-induction. (B) Response up to 6 h post-induction.

the CAT-specific transcript levels were increased in ppGpp-less strain only when cells were growing rapidly (as in LB medium), but not in slower growing M9 medium. Thus, eliminating intracellular ppGpp appears to have two effects on the production of CAT—an increase in transcript levels during exponential growth and enhancements in post-transcriptional processes during restricted growth.

CONCLUSIONS

We have investigated the effects of eliminating ppGpp on recombinant protein synthesis in both batch and fed-batch cultivations. Results from batch cultivations indicated a 5-fold increase in CAT production in the ppGpp-less strain when grown in LB medium and a 2-fold increase when grown in M9 medium. When the ppGpp-less strain was grown in LB medium, the rapid increase in CAT production during exponential growth was accompanied by a 2-fold increase in CAT mRNA level. This increase in transcript levels may in part explain the difference in CAT production during exponential growth. Many other cellular processes, such as plasmid replication, translation, and proteolysis, may also be responsible for this enhancement. However, plasmid copy effect is not expected to be significant. As reported by Herman and Wegrzyn (1995) the plasmid copy number of ColE1-based replicon was not affected significantly by different ppGpp levels. Furthermore, no major difference in translation or proteolysis is expected during

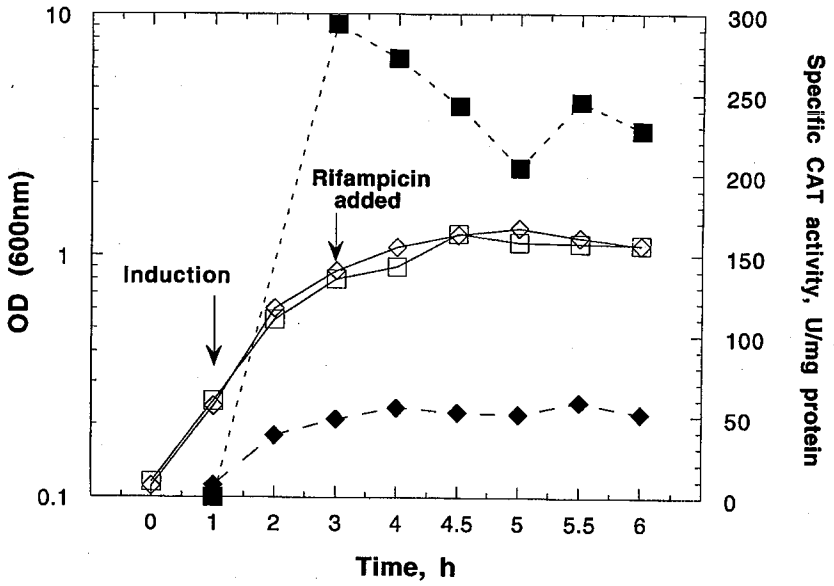

Figure 6. Effect of rifampicin treatment on CAT expression in CF1648:pKC6 and CF1693:pKC6 cells. Cells were induced with IPTG (to 1 mM), then with rifampicin as described in materials and methods. Cell growth rate [◇-CF1648:pKC6, □-CF1693:pKC6] and CAT activity [◆-CF1648:pKC6, ■-CF1693:pKC6] are shown.

exponential growth since even wild-type cells do not synthesis an appreciate level of ppGpp.

In contrast, when cells were grown in M9 medium an increase in CAT production was observed in the ppGpp-less strain only during the slow-growing regime, where no difference in CAT transcript levels were detected. It is likely that a different mechanism in the post-transcriptional machinery, such as proteolysis or translation, may be responsible.

Similar enhancements in CAT production were observed in fed-batch fermentations. CAT accumulation was about 20-fold higher in the ppGpp-less strain during the fed-batch phase. This is indicative of the importance of intracellular ppGpp on recombinant protein synthesis especially during slow-growing fed-batch phase. We are currently investigating the possible effects of varying intracellular ppGpp concentrations on recombinant protein synthesis during both exponential growth and restricted growth.

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